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THE TAKATA-ARA REACTION*

I. CLINICAL SIGNIFICANCE

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DURING the past year, the Takata-Ara test was performed on a series of 75 cases. Our purpose was to determine what diagnostic value the test has in cases of liver disease. With that in view, the reaction was determined in a group of patients with outspoken liver disease, and in a group in which there was no clinical reason to suspect any liver involvement. The latter were intended to serve as controls.

HISTORY

In 1925 Takata[1] devised a test depending on a colloidal reaction which he felt was of value in differentiating between lobar and bronchopneumonia. In the same year Takata and Ara,[2] using cerebrospinal fluid, reported further experiences with the test, which suggested its use in differentiating between meningitis and syphilitic involvement of the central nervous system. These investigators attributed the flocculation which occurs in the positive test to a change in the relationship of the albumin and globulin in the serum or spinal fluid.

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In 1929 Nicole,[3] under the direction of Staub, showed that a positive Takata-Ara reaction in spinal fluid occurs when the albumin-globulin ratio is changed, particularly the globulin. In 1924 Adler and Strauss[4] had shown that in cases where there was liver damage, the relationship of the serum albumin and globulin was disturbed. Having these facts in mind, Staub[5] suggested that the Takata-Ara reaction might be of value as a diagnostic measure in liver disease, and actually found the test positive in all cases with severe parenchymatous liver damage. Influenced by Staub's suggestion, Jezler[6-8] performed the test on serum and ascitic fluid, and reported positive results in cases of severe parenchymatous disease of the liver, particularly cirrhosis.

Since then numerous conflicting reports have appeared endeavoring to assay the value of the test as a diagnostic measure in liver disease. Greatest attention has been given to the results of the reaction in cases of cirrhosis. Most investigators, among them Staub,[5, 9] Skouge,[10] Oliva and Pescarmona,[11] Schindel and Barth,[12] and Heath,[13] have obtained positive results in a large percentage of their cirrhosis cases. Takata,[14] in a monograph written in 1935, found that of all the cases reported in the literature the test was positive in 90.1 per cent and negative in 9.9 per cent of cases of liver cirrhosis. Similarly, Kirk[15] in 1936 collected from the literature 375 cases of liver cirrhosis of which 315 gave a positive Takata-Ara reaction.

On the basis of reports similar to these, some have concluded that the test is fairly specific for cirrhosis of the liver. However, most of these authors agree that in the early stages of the disease the test is frequently negative. Crane[16] found the test to be highly specific in cirrhosis, but also in cases of acute yellow atrophy; and many authors[9-11, 17] have reported positive results in various other diseases associated with liver damage. On the other hand, Neuweiler[18] found that in toxemia of pregnancy, even when there was severest liver change, the test was always negative. Fulde[19] had similar results. Ragins,[20] however, did obtain positive results in cases of toxemia of pregnancy.

Oefelein,[21] Ragins, and others report positive reactions in cases of liver disease in which the degree of liver damage is not always extensive, such as malignant involvement of the liver, stasis due to cardiac failure, hepatocellular (catarrhal) jaundice, and liver damage secondary to cholelithiasis with obstruction.

Different results have been obtained in carcinomatous involvement of the liver. Schindel and Barth, van Ginkel,[22] Magath,[23] and Kirk have found that the test may be positive in liver malignancy. On the other hand, Jezler, Crane, and Hugonot and Sohier[24] obtained negative results in this condition. Fulde claims that the test is *always* negative in carcinoma, even with extensive involvement of the liver where very little functioning liver tissue remains. He concludes, therefore, that the test is of absolute value in differentiating between carcinoma involving the liver and cirrhosis. Since this differential problem not infrequently confronts the clinician, such claims for the test become of great importance and demand very critical consideration.

METHOD AND INTERPRETATION OF REACTION

We employed Crane's modification of the method used by Jezler. In every case the test was performed on the serum within one to two hours from the time the blood was drawn, although according to Jezler,[7] Crane, and Kirk serum kept in the refrigerator or preserved with toluol or thymol can be used after several days or even weeks.

One cubic centimeter of 0.9 per cent solution of sodium chloride is placed into each of six small tubes. To the first tube 1 c.c. of serum is added. After mixing with the saline, 1 c.c. of the mixture is removed and added to the saline in the second tube. This procedure is continued throughout the six tubes, 1 c.c. of the mixture from the last tube being discarded. The six tubes now contain serum dilutions of 1:2 to 1:64. To each of the tubes there is then added 0.25 c.c. of 10 per cent sodium carbonate. After shaking, 0.15 c.c. of 0.5 per cent mercuric chloride is added. The tubes are thoroughly shaken and examined after five minutes and again after twenty-four hours. A positive reaction is indicated by a pearly flocculum. Occasionally a reddish precipitate occurs which is not a true flocculum and should not be considered as representing the specific reaction.

Our manner of interpreting the results was as follows:

1. Strong positive.—A definite flocculation in 4 or more tubes, or in 3 tubes at least one of which shows a flocculum occupying about one-third of the column.

2. Positive.—A definite but small flocculation in 3 or 4 tubes.

3. Weak positive.—A definite but minimal flocculation in no more than 3 tubes.

All others were considered negative.

There is a difference of opinion among various authors as to the extent to which the readings of the reaction may be subdivided. Heath divides the readings into five groups. Magath also lists five groups, but actually considers those falling into the group he terms "suspicious" as negative. He states that "from a technical standpoint it is impossible to identify the test accurately as positive unless a degree of precipitation is present sufficiently great to leave no doubt about the positiveness of the reaction." In our series, we regarded as negative a minimal flocculation in one or two tubes, which Magath considers weakly positive.

RESULTS

The test was performed 82 times on the 75 cases. Table I is a summary of the results obtained in the entire series. Of special interest to us were the results obtained in liver cirrhosis, malignancy involving the liver, and liver enlargement due to cardiac failure. Of the 7 cases of clinical cirrhosis, 2 were regarded as of syphilitic origin. In the other 5 cases, the diagnosis of true Laënnec cirrhosis was made. In one of the cases of Laënnec cirrhosis our first

TABLE I

RESULTS OF THE TAKATA-ARA REACTION IN 75 CASES

DISEASE	NO. OF CASES	NO. OF TESTS	POSITIVE REACTIONS STRONG	POSITIVE	WEAK	TOTAL POSITIVES	NEGATIVE REACTIONS
Cirrhosis:							
Laënnec	5	8	4	3	0	7	1
Syphilitic	2	4	1	2	0	3	1
Carcinoma of the liver	7	8	3	1	4	8	0
Parenchymatous liver damage:							
Hepatocellular jaundice	4	4	0	0	1	1	3
Syphilitic hepatitis	1	1	0	0	1	1	0
Acute toxic hepatitis	1	1	0	0	1	1	0
Liver abscess	1	1	0	0	0	0	1
Obstructive jaundice:							
Stricture of common duct	1	1	0	1	0	1	0
Calculus in common duct	3	3	0	0	0	0	3
Adenocarcinoma of common duct	1	1	0	1	0	1	0
Adenocarcinoma of pancreas	3	3	0	0	1	1	2
Hepatomegaly of undetermined origin	8	8	0	2	1	3	5
Cardiac decompensation with permanent liver enlargement:							
Arteriosclerotic and/or hypertensive heart disease	8	8	0	0	2	2	6
Rheumatic	2	2	0	0	1	1	1
Thyrotoxic	1	1	0	0	0	0	1
Miscellaneous	27	28	0	6	1	7	21
Totals	75	82	8	16	13	37	45

result was negative. Exactly one month later the reading was strongly positive. In one case of syphilitic cirrhosis the test was at first negative, but 4 days later it was strongly positive.

In 6 of the cases with ascites, the Takata-Ara reaction was performed on the ascitic fluid. Two of these gave a positive reaction in both the blood and ascitic fluid. In two other cases, although the ascitic fluid gave a positive reaction, the degree of flocculation was not as marked as in the blood. In one instance, the reaction in the blood was strongly positive, the ascitic fluid negative. In the sixth of this group, the blood was at first negative, and at that time the ascitic fluid was also negative. On a second examination, when the reaction was strongly positive in the blood, the ascitic fluid was still negative. On a third determination the reaction in the blood was still positive and that in the ascitic fluid had become weakly positive (see Table II).

From Table I it will be noted that all of the 7 cases of liver malignancy gave positive reactions. Of the 8 tests done, 3 were strongly positive, 1 positive, and 4 weakly positive. In this group the diagnosis was confirmed in 3

TABLE II

RELATIONSHIP BETWEEN TAKATA REACTION IN BLOOD AND ASCITIC FLUID

CASE NO.	TAKATA IN BLOOD	TAKATA IN ASCITIC FLUID
3	+++	++
7	+++	0
10	0	0
	+++	0
	+++	+
15	++	++
68	++	++
70	++	+

+++=strong positive ++=positive +=weak positive 0=negative

by autopsy or operation. In the other 4, the fact that the site of a primary lesion was known and the hard nodular state of the liver left little doubt as to the diagnosis of carcinoma of the liver.

Under the head of parenchymatous liver disease, it will be noted that of the 4 cases with hepatocellular (catarrhal) jaundice, one gave a positive reaction. Each of our 2 hepatitis cases, one syphilitic and the other acute toxic, gave positive reactions.

We had in our series 11 cases of long-standing liver enlargement in cardiac patients who were chronically decompensated. These may justifiably be assumed to be cases of cardiac cirrhosis. Of this group, 3 gave positive reactions, 8 negative.

It is of interest to note that of our 27 miscellaneous cases, serving as controls, 6 gave a positive reaction. The diagnoses in these cases were: senile cataract, chronic myelogenous leucemia and bronchopneumonia, chronic glomerular nephritis, peritoneal carcinomatosis, lobar pneumonia and asthma, chronic adnexal disease. In none of these was there any clinical evidence of liver cell damage, although in the first three mentioned, the liver was enlarged. However, it should be noted that there were 3 other cases with liver enlargement in this miscellaneous group in which the Takata reaction was negative.

COMMENTS

Our findings are in accord with those of most investigators who claim that the test is positive in advanced cirrhosis, but may be negative in early cirrhosis. However, we must point out that in only two of our cases of cirrhosis did we obtain a negative reaction first and a positive reaction later. But, since the time interval between the two tests was in the one instance a period of one month and in the other only four days, it is unwise to attempt to attribute this change to a progression of the liver disease from an early to an advanced stage. Furthermore, in both instances, when the test was negative, the patient clinically showed evidences of advanced hepatic cirrhosis. We had a further experience of obtaining a weak positive reaction in a patient with a peptic ulcer, in whom the reaction was negative one week later. This difference in the results obtained would rather indicate the unreliability of one determination in any suspected case. Fulde reports a case in which this is demonstrated even more clearly. The case, according to clinical criteria, was definitely one

of liver cirrhosis. The test performed on two successive days was negative the first day and weakly positive the next. As a diagnostic test in cirrhosis, therefore, the Takata-Ara reaction is of little value, since by the time the reaction becomes positive the diagnosis can usually be made clinically.

Even with this fact in mind, the test would still be of considerable value if the contention of Fulde and others could be substantiated that it is negative in metastatic liver involvement and therefore can be used to differentiate between cirrhosis and carcinoma, where this differentiation presents difficulties clinically. In our series, however, the test was positive in all cases in which there was any degree of certainty about the diagnosis of carcinoma. We can, therefore, not subscribe to the view that the test is of differential diagnostic value. The results of others would also indicate that the test may be positive in malignancy. In Kirk's series, for example, of 5 cases with liver metastases, 3 gave a positive reaction. Of the 133 cases of tumor of the liver which he gathered from the literature, over 30 per cent gave a positive Takata-Ara reaction. Magath similarly reports 7 positive reactions out of 9 cases of carcinoma involving the liver. Schindel and Barth obtained 8 positive reactions in 9 cases of liver malignancy.

Along with Fulde, we have found that a small percentage of cases with liver enlargement due to cardiac failure show a positive Takata-Ara reaction. This has been the experience of many investigators. In the 3,583 cases collected from the literature by Kirk, 122 were cases of passive congestion of the liver and 20 per cent of these showed a positive Takata-Ara reaction.

The results in other parenchymatous affections of the liver are inconstant. The test is positive in some cases of hepatocellular jaundice and negative in others. In obstructive jaundice we found the test negative 5 times and positive in 3 cases. Magath reports a negative reaction, except where extensive liver damage was present. We cannot agree with Oefelein that the test is such an extremely sensitive indicator of the condition of the liver parenchyma that it will register even small changes. By plotting the amount of flocculation observed in each tube, he obtains various types of curves and feels that the type of curve is indicative of either progress or regression of the disease process in the liver. Magath finds that the degree of positiveness is not well correlated with the degree of liver disease and states that "it is evident that the subdivisions of the test are too fine for clinical use."

The fact that positive reactions were obtained in 6 cases where there was clinically no liver involvement is significant in that it supports the view that the test is by no means a specific one. It was found by others to be positive in a variety of nonhepatic diseases, for example, syphilis,[22, 25] pulmonary tuberculosis,[26] glomerular nephritis,[27] infectious diseases,[20, 24] and protozoal infestations of the blood.[24] Four out of 33 patients with no evidence of liver disease in Magath's series gave a positive reaction, and Kirk obtained similar results in 7 out of 39 patients. Heath showed that 3 per cent of general medical cases in which liver damage was not diagnosed gave positive reactions. Findings such as these have led Jezler, Magath, Kirk, and many others to the conclusion that the test is not specific for cirrhosis of the liver.

Some investigators who have performed the test on the ascitic fluid as well as on the blood serum, feel that the reaction in both media runs parallel. Staub and Jezler[28] go further and claim that the test can be performed with equally reliable results on various body fluids, for example the contents of blisters, pleural exudate, and knee joint effusions (although they did not obtain positive results in the spinal fluid of cirrhotics even when the blood was positive). In those cases, however, in which we performed the test simultaneously on the ascitic fluid and blood serum, we frequently found a discrepancy between the reactions obtained in the two media.

CONCLUSIONS

1. The Takata-Ara reaction is positive in cases of advanced liver cirrhosis.

2. It is also positive in most cases of malignancy involving the liver.

3. The test is, therefore, not a satisfactory means of differentiating between cirrhosis of the liver and carcinoma involving the liver.

4. The test is frequently positive in severe liver damage and may be positive in mild parenchymatous disease of the liver and in cases where the liver is enlarged due to cardiac failure.

5. The test is occasionally positive in cases in which there is clinically no evidence of liver disease.

6. The Takata-Ara reaction cannot be regarded as a specific test for cirrhosis of the liver, but a negative reaction in any suspected case would tend to cast doubt on the diagnosis.

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THE TAKATA-ARA REACTION*

II. Mechanism—With Special Reference to the Influence of the Ammonia Blood Level

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SINCE the introduction of the Takata-Ara test there has been considerable speculation as to the mechanism of the reaction. The test depends on an alteration in the stability of a colloidal suspension of a mercury oxysol, resulting in the formation of a pearly flocculum.

Various explanations for this flocculation have been offered. The one most generally accepted is that held by the originators of the test, Takata and Ara,[1, 2] according to whom the formation of the flocculum depends upon an alteration in the protein constituents of the blood serum. Some investigators believe that the factor of primary importance is the lowered albumin, while others take the view that the elevated globulin is the responsible agent. More recently the reaction has been attributed to various other factors, for example, the total proteins, fibrinogen, certain fatty acids, heparin, the hydrogen ion concentration, and the ammonia level in the blood.

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In 1935 Staub and Jezler,[3] in order to prove that the result of the Takata-Ara reaction is dependent upon the colloid make-up of the serum, performed an interesting series of experiments. They passed Takata-positive sera through Bechhold filters and found that the filtrate gave a negative reaction, while the residue continued to be positive. They concluded from this that the flocculation reaction was dependent upon the colloidal residue. Further, they warmed Takata-positive sera till clot formation occurred and then filtered and obtained a negative reaction in the filtrate. They also found that a pure 2 per cent globin solution, prepared from ox blood, always gave a positive reaction.

The evidence for the claim that an altered albumin-globulin ratio is responsible for the Takata-Ara reaction comes from various sources. Takata, Nicole,[4] and Jezler[5] showed that a positive reaction was obtained in cases in which this shift in the albumin-globulin ratio occurred. Jezler found an increased globulin in all of his Takata-positive sera. Jezler and Mezey[6] further demonstrated that in almost all positive cases there was an elevation of the tryptophan content of the serum above the upper limit of normal. Since globulin contains three times as much tryptophan as does albumin, they concluded that this was corroborative evidence of an elevation of the globulin fraction. This is stressed by most investigators who subscribe to the theory that the altered albumin-globulin ratio plays an important rôle. On the other hand, Tannenholz[7] feels that the albumin serves as a protective colloid to hinder the formation of a mercury precipitate in normal serum. Oliva and Pescarmona[8] from their work also concluded that the reaction depended on a diminution in the protective action of serum albumin. Abrami and Robert-Wallich[9] and most of the Italian school take the same view.

Adler,[10] in a monograph on plasma and serum, quoted the normal albumin-globulin figures obtained by various investigators in a large variety of animals. These showed a wide range of normal values. Staub and Jezler performed the Takata-Ara reaction on a small group of these animals and, using Adler's figures, demonstrated a direct relationship between the degree of positivity of the Takata-Ara reaction and the relative globulin content of the blood in all but the frog. Their results are so striking that their table is reproduced here (Table I).

TABLE I

ALBUMIN-GLOBULIN RATIO AND TAKATA REACTION IN VARIOUS ANIMALS

ANIMAL	TAKATA-ARA REACTION	ALBUMIN-GLOBULIN RATIO
Guinea Pig	0	2.4/1
Pig	0	1.5/1
Man	0	1.5/1
Sheep	(+)	?
Cow	+	0.8/1
Horse	++	0.6/1
Frog	0	0.2/1

Most of the cases reviewed by Staub and Jezler did show the albumin-globulin ratio below normal but only one-third showed a ratio of less than one. The recent investigations of Magath[11] and Kirk[12] support the view that the shift in the albumin-globulin ratio is in large measure responsible for the positive

reaction obtained. Magath found such a shift in 12 out of 21 cases with a positive Takata-Ara reaction. Kirk obtained positive Takata-Ara reactions in 13 out of 18 cases showing altered ratio. The latter goes further and agrees with Jezler[5] and Lazzaro[13] that there is a definite relationship between the positive Takata-Ara and an increase in the globulin fraction. He found that in only 3 of his 22 cases with a positive reaction was the globulin fraction less than 3, and that of 20 sera in which the globulin was 3 or over only one gave a negative Takata-Ara reaction.

Some investigators have presented evidence which casts some doubt on the existence of a parallelism between the Takata-Ara reaction and the altered albumin-globulin ratio. In 2 of Magath's cases, despite the reversal of the albumin-globulin ratio, the reaction was negative. Lazzaro also occasionally obtained a negative Takata-Ara reaction with a low ratio. Skouge[14] found this to be true even more often. Schindel and Barth,[15] in only 7 of 21 positive sera, obtained albumin-globulin ratios of less than 1. Fulde[16] feels that there is no parallel between the result of the reaction and the degree of increase in globulin and suggests that the shift in the albumin-globulin is only one of the factors responsible for a positive Takata-Ara reaction.

Abrami and Robert-Wallich frequently found a decrease in the value of the total protein in Takata-positive sera. However, Schindel and Barth failed to find a relationship between the Takata-Ara reaction and the total serum protein. Hugonot and Sohier[17] maintain that a positive Takata alone is not as indicative of liver damage as when associated with a diminution in the total serum protein. Staub and Jezler deny this contention, and Fiessinger and Gothie[18] failed to observe either constant or significant alterations in the serum protein in dogs following Eck fistulas or hepatectomy.

In an effort to determine the influence of proteinemia on the Takata-Ara reaction, Staub and Jezler performed several experimental studies. They endeavored to bring about a positive reaction in people with healthy livers by means of a diet rich in protein. Five subjects were fed a breakfast containing 70 to 100 grams of protein. Four hours later the blood was examined with negative results. They also attempted to reverse positive Takata reactions by feeding abundant carbohydrates. However, it appears questionable whether a diet containing such quantities of protein would be adequate to effect any appreciable change in the total protein of the serum of normal individuals. Magath states that there is no evidence to indicate that changes in the total protein can be correlated with the Takata-Ara reaction. He suggests that the fibrinogen content of the serum may influence the reaction, thus accounting for Takata's positive results in cases of pneumonia.

The suggestion that low fatty acids are responsible for the Takata-Ara reaction has been under investigation. This work was initiated by the findings of Kallós-Deffner.[19] In 1933, working with rabbits, she demonstrated that during starvation, or when the animals were kept in a rarefied atmosphere for a long time, large quantities of acetone and B-hydroxybutyric acid appeared in the blood. Her further studies showed that while this ketonemia was present the Takata-

Ara reaction was positive and that with its disappearance the reaction became negative. Schindel[20] found that the addition of low fatty acids to Takata-negative sera rendered the reactions positive and expressed the belief that positive reactions were dependent upon the presence in the blood of such fatty acids, occurring as intermediate products of metabolism in liver disease. Ucko,[21] and Takata[22] himself do not subscribe to this view. The former feels that the flocculation following the addition of low fatty acids is merely due to acidification and disturbance of the mercury salts. Staub and Jezler also reject Schindel's contention. They determined the ketone bodies in the blood of many diabetics with marked ketonuria and compared their findings with the results of the Takata-Ara reaction. They could not find the slightest relationship between the two. The fact that cases in diabetic coma never gave a positive reaction was to them further strong evidence against the fatty acid theory.

Zirm,[23] Medvei and Paschkis,[24] and Ragins[25] have found that the addition of heparin to Takata-positive sera rendered them negative. This would seem to indicate that this product of the healthy liver inhibits the flocculation and is present in inadequate amounts when the liver is diseased. On the other hand, the experiments of Jürgens[26] may be taken as contrary evidence. He found that extirpation of the liver in geese did not produce positive Takata-Ara reactions, even though all evidences of fatal liver insufficiency developed. Furthermore, in evaluating any experimental work involving the use of heparin, the fact stressed by Fuchs[27] should be borne in mind. He called attention to the impurity of the available heparin preparations and cautioned against drawing false conclusions from the results obtained with them. To date, insufficient work has been done to substantiate the claim that heparin is an important factor in the mechanism of the Takata-Ara reaction.

The recent work of Oefelein[28] opened a new line of investigation into the mechanism of the Takata-Ara reaction. Knigge,[29] and Röhrs and Kohl-Egger[30] had claimed that the important factor in the reaction was the hydrogen ion concentration and the salt content of the serum. Their results showed that the flocculation was less likely to occur when the medium tested was strongly acid, and that, when the alkaline content was high, flocculation was more pronounced. It had also been shown that the addition of a small amount of ammonium sulfate to the Takata-Ara reagent always produced a white precipitate. Accepting these findings and the contention of von Schroeder[31] and Thannhauser[32] that the liver forms urea from the ammonia in the circulating blood, Oefelein concluded that the amount of ammonia ion present in the blood serum would be a measure of the ability of the liver to form urea, the higher the ammonia level the poorer this function. Therefore, apparently with the following in mind: first, that the Takata-Ara reaction is a test of liver function; second, that the ammonia level in the blood is an index of one of the functions of the liver; and third, that the Takata-Ara reaction is determined by the presence of the ammonia ion, Oefelein studied the ammonia level in the blood serum and its relation to the Takata-Ara reaction.

Using the Folin-Denis[33] method of determining preformed ammonia, but employing blood serum instead of whole blood, he found that in all cases with a

positive Takata-Ara reaction the ammonia level was greatly increased. He obtained ammonia values of 1.02 to 2.4 mg. per cent where the reaction was distinctly positive; 0.76 to 0.85 mg. per cent in cases with a mildly positive reaction; and 0.2 to 0.58 mg. per cent in all cases in which the reaction was negative. He concluded that there was a definite parallelism between the degree of positivity of the Takata-Ara reaction and the level of the ammonia in the blood serum. Impressed by these striking findings, we set out to determine the ammonia content of the blood serum and the Takata-Ara reaction in a series of cases.

METHOD AND PROCEDURE

Our first problem was to select the best available method of determining the ammonia level in the blood. We made a careful study of the various methods appearing in the literature. This led to the observation that the procedure described by Folin[34] in 1932 was the least complicated and the one used by most recent investigators. We chose this method for these reasons and also because it was similar to the one employed by Oefelein in his work. After performing the test many times, we found that the substitution of titration for the colorimetric determination originally described gave results which to us appeared more accurate.*

Our determinations were invariably started within one and one-half hours of the removal of the blood from the vein. Folin[35] and others[36, 37] call attention to the fact that blood on standing rapidly decomposes with the formation of ammonia. Therefore any determination of ammonia in the blood, in order to be accurate, should be done within two or at most three hours. To exercise the greatest possible precaution against alteration of the blood ammonia following venepuncture, we used the method of collecting specimens described by Atchley, Loeb, and others,[38] which they employed for the purpose of assuring minimal changes of the pH level in blood serum. The blood was drawn from a cubital vein into a syringe well lubricated with mineral oil and delivered under a two-inch column of oil into a centrifuge tube. This was allowed to stand for exactly one hour, after which the clot was separated with a glass rod and the tube centrifuged. The serum was then drawn off from beneath the mineral oil column and determinations of the ammonia content of the serum and the Takata-Ara reaction were immediately begun. The method of performing the Takata-Ara test and the manner of interpreting the results were the same as those described in an earlier communication.[39]

The material upon which this report is based is to a large extent drawn from our series in which the clinical significance of the Takata-Ara reaction was studied. This consisted of sera from a large variety of cases. Some of the cases had outspoken cirrhosis of the liver, others had various types of parenchymatous liver disease, and a third group had no obvious involvement of the liver. We

*Oefelein employed the method devised by Folin and Denis[33] with the modifications of Thierfelder as described in Hoppe-Seyler and Thierfelder's Handb. physiol. u. path. chem. analyse (ed. 9, Berlin, 1934, Julius Springer). We also used methyl red as indicator. Since the quantities of ammonia were so small, we found it preferable to use a weaker acid in the receiving bottle and therefore substituted 0.01 normal sulphuric acid for the 0.1 normal acid. During the process of aerating the serum, a great deal of foaming occurred which could be checked by the addition of a few drops of amyl alcohol.

could, therefore, reasonably anticipate Takata-Ara reactions ranging from strongly positive to negative. The entire series numbered 56 cases.

RESULTS

In order to present the results most clearly, we have in Table II divided our cases into four groups according to the degree of positivity of the Takata-Ara reaction. In each case we have listed the ammonia content of the blood serum. It will be noted that the ammonia level ranged from 0.136 to 0.442 mg. per cent in the cases giving a strongly positive reaction; from 0.170 to 0.680 mg. per cent in those with a positive reaction; and from 0.170 to 0.849 mg. per cent in those giving a weak positive reaction. In the group which showed a negative Takata-Ara reaction, the ammonia level varied from 0.034 to 0.786 mg. per cent.

In a small group, 28 cases, we also studied the relationship of the Takata-Ara reaction to the total proteins and serum albumin and globulin. The results of this study are shown in Table III.

COMMENTS

It can readily be seen from Table II that our tests failed to show any relationship between the level of the ammonia in the blood serum and the degree of positivity of the Takata-Ara reaction. As a matter of fact, some of the highest ammonia figures we obtained were in the group which gave negative reactions. Our findings, therefore, fail to substantiate the claims made by Oefelein. Furthermore, it should be noted that our highest figures are considerably lower than the high readings obtained by this author.

Staub and Jezler criticize Oefelein's assumption that the ammonia radical plays an important rôle in the production of a positive Takata-Ara reaction. They point out the fact that the phenomenon observed when ammonium sulfate is added to Takata-Ara reagent is a precipitation and not a true colloidal flocculation, and hence the result cannot be interpreted as a positive Takata-Ara reaction. In addition, Oefelein's assumption that the ammonia level in the blood is an index of the degree of liver insufficiency is open to question. Although it is known that the liver forms urea from ammonia,[40] it does not follow that an elevated ammonia level in the blood necessarily points to a deficiency in liver function, since the formation of ammonia is apparently also a property of organs other than the liver,[41] and since ammonia is also absorbed in appreciable amounts from the intestine.[42] Another point which may be raised in evaluating Oefelein's work is the high ammonia figures reported by him. It is known that "the toxic properties of ammonia preclude the possibility of its existence in appreciable amounts in the circulating blood."[43]

Although we were aware of the possible objections to Oefelein's line of reasoning on physiologic grounds, we felt that these did not invalidate his experimental findings which so definitely pointed to an elevated ammonia level as a primary factor in the production of a positive Takata-Ara reaction.

It will be seen from Table III that in 13 of our 16 cases, where the reaction was positive, there was an alteration in the albumin-globulin ratio. (We re-

TABLE II

RELATIONSHIP OF TAKATA-ARA REACTION TO THE AMMONIA LEVEL IN THE BLOOD SERUM

CASE NO.	DIAGNOSIS	TAKATA-ARA REACTION	NH_3 IN MG. PER 100 C.C.
3	Laënnec cirrhosis	Strong positive	0.136
10	Syphilitic cirrhosis	Strong positive	0.340
25	Laënnec cirrhosis	Strong positive	0.442
10	Syphilitic cirrhosis	Positive	0.212
14	Senile cataract	Positive	0.170
15	Syphilitic cirrhosis	Positive	0.425
17	Laënnec cirrhosis	Positive	0.680
19	Chronic myelogenous leucemia	Positive	0.272
29	Metastases to peritoneum and omentum	Positive	0.510
38	Lobar pneumonia	Positive	0.374
40	Chronic adnexal disease	Positive	0.612
9	Hepatomegaly of undetermined origin	Weak positive	0.255
12	Acute hepatitis	Weak positive	0.612
13	Arteriosclerotic heart disease (decomp.)	Weak positive	0.340
16	Syphilitic hepatitis	Weak positive	0.170
34	Ca of gall bladder with liver metastases	Weak positive	0.340
37	Arteriosclerotic heart disease (decomp.)	Weak positive	0.442
42	Undiagnosed neoplasm of liver	Weak positive	0.408
44	Ca of ovary, metastases to liver	Weak positive	0.408
48	Adenocarcinoma of pancreas	Weak positive	0.849
56	Acute catarrhal jaundice	Weak positive	0.226
1	Acute catarrhal jaundice	Negative	0.714
2	Nonspecific colitis	Negative	0.068
4	Hypertensive heart disease (decomp.)	Negative	0.306
5	Acute coronary thrombosis	Negative	0.042
6	Arteriosclerotic heart disease (decomp.)	Negative	0.085
8	Hepatomegaly of undetermined origin	Negative	0.238
11	Cyst or tumor of the liver	Negative	0.340
18	Rheumatic heart disease (decomp.)	Negative	0.119
20	Lung abscess	Negative	0.187
21	Hypertensive heart disease (decomp.)	Negative	0.272
22	Desmoid tumor of abdominal wall	Negative	0.272
23	Arteriosclerotic heart disease (decomp.)	Negative	0.642
24	Thrombocytopenic purpura	Negative	0.408
25	Laënnec cirrhosis	Negative	0.034
26	Multiple liver abscesses	Negative	0.340
27	Acute catarrhal jaundice	Negative	0.736
28	Lymphosarcomatosis	Negative	0.786
30	Obstruction in common bile duct (stone)	Negative	0.509
31	Acute follicular tonsillitis	Negative	0.323
32	Gonococcal arthritis and prostatitis	Negative	0.425
33	Bronchopneumonia	Negative	0.255
35	Stone in common bile duct	Negative	0.187
36	Acute catarrhal jaundice	Negative	0.306
39	Arteriosclerotic heart disease	Negative	0.208
41	Thyrotoxic heart disease (decomp.)	Negative	0.374
43	Lobar pneumonia, rheumatic heart disease	Negative	0.442
45	Undiagnosed	Negative	0.340
47	Normal	Negative	0.762
49	Acute upper respiratory infection	Negative	0.544
50	Rheumatic heart disease (inactive)	Negative	0.750
51	Coronary sclerosis	Negative	0.476
52	Acute upper respiratory infection	Negative	0.442
53	Hypertensive heart disease	Negative	0.408
54	Pleural effusion	Negative	0.204
55	Coronary thrombosis	Negative	0.272
57	Bronchopneumonia	Negative	0.452
58	Pleuritis sicca	Negative	0.068

TABLE III

RELATIONSHIP OF THE TAKATA-ARA REACTION TO THE SERUM PROTEINS

CASE NO.	TAKATA-ARA REACTION	TOTAL PROTEIN	ALBUMIN	GLOBULIN	ALBUMIN-GLOBULIN RATIO
3	Strong positive	3.91	1.08	2.83	0.38
7	Strong positive	5.85	2.81	3.04	0.92
10	Strong positive	5.22	2.44	2.78	0.88
25	Strong positive	5.20	2.95	2.25	1.31
71	Strong positive	6.25	2.34	3.91	0.59
73	Strong positive	4.69	2.21	2.48	0.89
17	Positive	6.25	3.51	2.74	1.28
61	Positive	8.51	4.32	4.19	1.03
67	Positive	3.75	2.16	1.59	1.36
68	Positive	4.69	2.12	2.57	0.83
70	Positive	7.20	4.01	3.19	1.26
72	Positive	4.46	1.87	2.59	0.72
74	Positive	4.46	1.87	2.59	0.72
13	Weak positive	6.25	3.12	3.13	1.00
37	Weak positive	6.05	3.41	2.64	1.29
60	Weak positive	6.86	4.02	2.84	1.41
2	Negative	4.69	2.94	1.75	1.68
10	Negative	6.25	3.30	2.95	0.89
21	Negative	5.25	3.00	2.25	1.33
23	Negative	5.85	3.30	2.55	1.29
25	Negative	5.86	2.81	3.05	0.92
45	Negative	5.07	2.74	2.33	1.17
59	Negative	5.51	2.81	2.70	1.04
62	Negative	5.22	3.12	2.10	1.48
63	Negative	4.68	2.68	2.00	1.34
64	Negative	6.55	3.75	2.80	1.34
65	Negative	6.25	3.13	3.12	1.00
75	Negative	2.75	1.27	1.48	0.85

garded 1.3 as the lowest normal relationship of albumin to globulin.) In just 50 per cent of our positive cases was the ratio below 1. These findings are in agreement with those reported by other investigators. However, it must be pointed out that in 7 of the 12 negative reactions there was also an altered ratio, 25 per cent of these negative cases showing a ratio of below 1. In only 5 of the 16 positive cases was the globulin figure above 3. The 11 cases with positive Takata-Ara reactions in which the globulin was below 3 constitute a much larger percentage than that obtained by Kirk. Furthermore, in 2 of the 12 cases with negative reactions the globulin figure was above 3. Skouge, in a large group of noncirrhotics with extremely high globulin figures, obtained negative Takata-Ara reactions.

CONCLUSIONS

1. There is no relation between the ammonia content of blood serum and the Takata-Ara reaction.

2. In most cases with a positive Takata-Ara reaction there is an alteration of the albumin-globulin ratio.

3. The altered albumin-globulin ratio, although not the sole factor, is probably the most important one in the mechanism of the Takata-Ara reaction.

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ACTIVE IMMUNIZATION AGAINST TETANUS BY MEANS OF TETANUS TOXOID ALUM-PRECIPITATED REFINED

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BERGEY and Etris,[1, 2] Jones and Moss,[3] Gold,[4, 5] Hall,[6] McBryde,[7] and Cowles[8] have demonstrated that the injection into human beings of two doses of alum-precipitated tetanus toxoid is followed by the appearance in the blood of appreciable quantities of tetanus antitoxin. It has also been established that these injections can be given with safety and without the development of unpleasant reactions. The amount of tetanus antitoxin produced, and its persistence in the blood appear to be very variable. These two factors are not dependent upon race, sex, or age, but seem to be related to the individual constitution and the state of health of the host at the time of antigenic stimulation.

An analysis of the published data reveals several important questions which must be answered before active immunization against tetanus can be recommended as a routine procedure. 1. What is the minimum titer of tetanus antitoxin that will protect an actively immunized individual against infection with *Cl. tetani?* 2. What is the optimum dose of toxoid to be injected? 3. What interval of time should elapse between the two injections to obtain the best antitoxin reponse? 4. How soon after the course of immunization is completed does immunity develop? 5. How long does active immunity last? 6. If such an immunity is lost, how soon can the antitoxin titer be restored to a protective level following the injection of a third or subsequent doses of alum toxoid?

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In reference to the first question, two avenues of experimental approach exist: (1) protection tests in actively immunized laboratory animals, and (2) studies of antitoxin titers in passively immunized human beings. Although Sneath and his associates[9] submit that an actively induced resistance to infection by *Cl. tetani* as shown in guinea pigs is the most convincing criterion possible of attainment in the laboratory, one must always keep in mind the possibility that such findings are not directly transferable to man. Moreover, although the infective experimental dose is determinable, the amount of free toxin produced by the spores in the wound and responsible for the clinical picture of tetanus, is a variable factor beyond control.

Jones and Jamieson[10] found that guinea pigs that had received one or more injections of tetanus alum-precipitated toxoid and were injected within two months, were protected against a massive dose of tetanus spores which killed normal pigs in from eighty-eight to one hundred and twelve hours. With the infective dose used, these workers found that it required from two to three units of antitoxin per cubic centimeter of blood in the immunized pig to neutralize the toxin produced from the infection. In this regard, it is interesting to note that though some of the guinea pigs had as high as four units of antitoxin, they developed localized tetanus from which, however, they completely recovered. These workers also found that a massive dose of tetanus spores did not markedly accelerate the production of antitoxin in guinea pigs that had been previously immunized with alum tetanus toxoid. This observation destroys the hope entertained by some[7] concerning the possible accelerating effect to be exerted upon the antitoxin level of the blood of an immunized person by tetanus spores if present in a wound of such a subject.

Sneath, Kerslake, and Scruby[9] injected various dilutions of spore suspensions mixed with equal volumes of 50 per cent calcium chloride intramuscularly into normal animals in doses of 0.1 c.c. They found that more uniform results may be expected when the spore distribution in the test dose is of the order shown by dilution 10^{-11}. The computable number of spores, varying from 3 to 50 per dose, when combined with calcium chloride, induced tetanus and killed unprotected guinea pigs without an experimental constant interval of forty-eight to seventy-two hours. Upon the injection of this lethal spore dose into a group of 55 guinea pigs actively immunized by means of tetanus toxoid, they found that 80 per cent of the animals were completely protected, 11 per cent were partially protected, and 9 per cent died of tetanus. In a similar group of guinea pigs that received 1500 units of antitoxin intraperitoneally, immediately after injection of the lethal spore dose, 24 per cent were completely protected, 38 per cent were partially protected, and 38 per cent died of tetanus. From their studies on the actively immunized group, it would appear that in the guinea pig, the level of 0.01 unit of tetanus antitoxin per cubic centimeter of serum is a critical level in tetanus immunization. Above that level, in 45 animals 13 per cent showed signs of tetanus, from which they recovered, while the remaining 87 per cent were completely protected. Sneath and his co-workers could not explain why three animals, showing less than 0.01 unit, were com-

pletely protected, while seven showing antitoxin in excess of that level (two having more than 0.1 unit) were only partially protected. They feel that in view of these discrepancies, it may be reasonable to suggest the possibility that antitoxin per se is not the sole factor influencing protection against the lethal spore dose. They conclude that since infection with *Cl. tetani* is a localized process, it is probable that such an antitoxic level (0.01 unit) as will prevent the manifestations of tetanus in guinea pigs would also be sufficient to prevent the disease in man. The incidence of localized tetanus in 13 per cent of the immunized animals is, however, worthy of emphasis, since in the human being, one must strive for complete protection in all cases.

Cowles[8] carried out a similar type of experiment on actively immunized guinea pigs and mice. Upon injection of 100,000 tetanus spores and 10 mg. of calcium chloride contained in a 0.2 c.c. volume, into a group of 27 immunized pigs, he found them completely protected against the disease during a three months' follow-up period, while 26 out of the 28 controls died in less than forty-six hours with tetanus. Before injection, 1 animal had less than 0.2 unit of antitoxin, 24 had 0.20 to 1 unit, and 2 had more than 1 unit. A second group of immunized animals was similarly treated, except that the infective dose contained 25,000 tetanus spores. In this group, Cowles encountered the same apparent inconsistency noticed by Sneath in his experiments. Two pigs that had 0.10 unit of antitoxin per cubic centimeter and a third animal that showed 1 unit per cubic centimeter died of tetanus in from six to eleven days, while 2 animals that had 0.05 unit and 1 guinea pig that had 0.02 unit were completely protected against the disease. In a group of 8 mice, whose antitoxin titer ranged between less than 0.05 to more than 0.05 unit of antitoxin per cubic centimeter, 5 died of tetanus in from three to seven days, 2 were paralyzed but survived, and 1 was completely protected when injected with 5,000 tetanus spores and 5 mg. of calcium chloride in a 0.1 c.c. volume. The controls were all dead in forty-eight hours. A group of mice showing 0.10 unit or more were completely protected against an infection of 100,000 spores. From his animal experimentations, Cowles concludes that although it is probably impractical to define the minimum titer which will assuredly protect against tetanus, antitoxin values of 0.10 or 0.20 unit per cubic centimeter of serum can give a fairly certain protection in immunized guinea pigs and mice at the time of infection. He feels that though 0.10 unit is not sufficiently great to protect all animals against a maximum infection, it is probably much larger than is necessary to care for many infections resulting from wounds judged to be too slight for surgical attention, and probably large enough to care for the majority of injuries which receive surgical treatment. He asserts that pending the acquisition of more information, conservative opinion may demand the maintenance of such a titer in cases where much reliance is to be placed on the immunity.

Bergey and Etris[1, 2] have suggested drawing an inconclusive analogy with diphtheria, that 0.01 unit may be satisfactory for protection. In a later publication[11] the same authors maintain that 0.01 unit is "the least amount of antitoxin necessary for protection and is based on the finding in the serum of

TABLE I

GROUP A

TETANUS TOXOID, ALUM PRECIPITATED, REFINED NO. 76812-1

Two doses, 0.5 c.c. each, given 645 days apart.

Titer expressed in units of tetanus antitoxin per cubic centimeter of blood serum.

CASE	CONTROL TITER	DAYS AFTER FIRST DOSE TITER		DAYS AFTER SECOND DOSE TITER							
6611	0.003	24 days 0.003	645 days +0.003 −0.01	34 days +0.25	90 days +0.25	184 days +0.25	284 days +0.25	365 days +0.25	455 days +3.0 −5.0	578 days 3.0	679 days 2.0
6617	0.003	24 days 0.003	645 days 0.003	32 days +0.25	89 days +0.25	200 days +0.10 −0.25	285 days 0.25	365 days +0.10 −0.25	455 days 0.25	687 days 0.10	
6644	−0.003	26 days −0.003	644 days −0.003	32 days +0.25	90 days +0.25	184 days 0.10	284 days +0.01 −0.10				
6657	+0.003	23 days +0.01 −0.10	644 days 0.003	32 days +0.10	90 days +0.25	184 days 0.10	290 days +0.01 −0.10				
6645	−0.003	23 days −0.003	644 days 0.003	32 days +0.25	90 days +0.10 −0.25	184 days +0.01 −0.10	284 days +0.01 −0.10				
6649	−0.003	26 days −0.003	644 days −0.003		90 days 0.10	185 days +0.01 −0.10	284 days +0.01 −0.10				
6643	−0.003	23 days −0.003	644 days −0.003	33 days 0.25	89 days +0.10 −0.25	184 days 0.01					
6642	−0.003	23 days −0.003	644 days −0.003	32 days +0.10 −0.25	90 days +0.01 −0.1	184 days 0.01	284 days 0.01				
6614	+0.01 −0.10	24 days +0.01 −0.10	645 days +0.01 −0.10	32 days 0.25	90 days +0.01 −0.10	172 days +0.01 −0.10	284 days −0.10				
6618	−0.003	24 days −0.003	645 days −0.003	32 days 0.10		171 days +0.01 −0.10	318 days −0.01	529 days +0.01 −0.1			
6636	0.003	24 days −0.003	645 days −0.003	32 days 0.25							

\+ More than
− Less than

the person who showed least response to the antigenic effect of the toxoid one year after the second dose. This individual had 0.01 of a unit of tetanus antitoxin." This finding cannot, however, be accepted as indicating the protection level against infection.

Our studies on passive immunization confirm Cowles' experimental findings. Prophylactic passive immunization with 1500 units of tetanus antitoxin has been successfully used to prevent lockjaw following injuries in both war and civil life. Titrations following the injection of such a prophylactic dose[4] reveal the presence of 0.1 to 0.25 units of antitoxin per cubic centimeter of blood serum. Hence, to be of value, active immunization must at least produce 0.1 unit of antitoxin per cubic centimeter. This minimal protective value does not appear to us to be too high, since we have on several occasions encountered control values of more than 0.01 unit of antitoxin in persons who were never immunized passively or actively, and who would consequently be considered susceptible to tetanus. Until direct proof exists that a lower antitoxin level is sufficient to prevent tetanus, one must insist on the presence of 0.1 unit of antitoxin per cubic centimeter of blood serum in order to consider an actively immunized person protected against this disease.

From our experience with human beings, we feel that a 1 c.c. dose of tetanus toxoid is more effective than smaller quantities of this antigen. We have also found[4] that when the two doses are given close together (one month or less), the antitoxin titer that follows is not as high as when they are injected at an interval of ninety days. Bergey and Etris[12] had a similar experience. McBryde[7] injected the two doses seventy-three days apart. Hall[6] allowed six weeks to elapse between the two injections. Jones and Moss[3] administered the two doses four weeks apart. Cowles[8] allowed an interval of eleven weeks between the two doses.

In most individuals the first injection of toxoid serves to prepare the host so that after a suitable interval of time, the administration of the second dose is followed by a rather prompt appearance in the blood of an increased amount of tetanus antitoxin. We have stated that this interval should preferably be three months. Actually, how long can this interval be?

Fifty adult workers (group A) were immunized with two 0.5 c.c. doses of Mulford tetanus toxoid alum-precipitated refined lot No. 76812-1, given six hundred and forty-five days apart. Six weeks after a single injection of 0.2 c.c., 1.0 c.c., and 2.0 c.c. of this toxoid was given to three groups of guinea pigs, respectively, their blood serums were tested according to the standard procedure of the National Institute of Health. The pooled serums of the 0.2 c.c. group showed 5 units of antitoxin. The 1 c.c. group showed 2 units, while the guinea pigs injected with 2 c.c. of this antigen had an antitoxin content of 3 units. These tests should not be interpreted as indicating that the smaller amount of tetanus toxoid constitutes an optimum dose.

The follow-up results on 11 members of this group A are given in Table I. Case 6614 is interesting because his control titer was greater than 0.01 unit per cubic centimeter. He had never been passively immunized, and the values ob-

TABLE II

GROUP B

TETANUS TOXOID, ALUM PRECIPITATED, REFINED NO. 76812-1

Two doses, 0.5 c.c. each, given two years apart.

Titer expressed in units of tetanus antitoxin per cubic centimeter of blood serum.

CASE	CONTROL TITER	DAYS AFTER FIRST DOSE TITER			DAYS AFTER THE SECOND DOSE TITER																	
		30	48	730	1	3	5	6	7	8	9	10	11	12	14	30	90	180	270	365	485	580	675
6510	−0.003	−0.10	−0.10	−0.10		−0.10									+0.25								
6511	−0.003	−0.10	−0.10	−0.10		−0.10									+0.25								
6512	−0.003	−0.10	−0.10	−0.10		−0.10									+0.25								
6530	−0.003	−0.10	−0.10	−0.10		−0.10									+0.25								
6612	−0.003	−0.10		−0.10							0.25			+0.25	+0.25	+0.25	+0.25	+0.25	+0.25	+0.25	0.25	0.25	
6623	−0.003	−0.10		−0.10	−0.10			−0.10			−0.10				+0.25	+0.25	+0.01	+0.01			0.01		
6714	−0.003	−0.10		−0.10	−0.10	−0.10		−0.10		+0.10			+0.25		+0.25	+0.25	+0.25	+0.01					
										−0.25								−0.10					
6682	−0.003	−0.10		−0.10		−0.10		0.25				+0.25			+0.25	+0.25	0.25	+0.10	0.25	+0.10	0.10	+0.10	
																		−0.25		−0.25		−0.25	
6683	−0.003	−0.10		−0.10			−0.10				+0.25				+0.25	+0.25		+0.25	+0.25	0.25		−0.10	
6639	−0.003	−0.10		−0.10	−0.10				−0.10						+0.25	+0.25	+0.25	+0.25	+0.25	+0.25	+0.25	+0.10	
																					−0.50	−0.25	
6716	−0.003			−0.10	−0.10	−0.10	−0.10		+0.10			+0.25			+0.25	+0.25	+0.25	+0.25	+0.25	+0.25	+0.5	+0.25	0.25
									−0.25											−2.0	−1.0	−0.5	

tained were checked repeatedly. The same titer was found in this case after the first injection of toxoid, but it went up in the expected fashion after the second dose. We have encountered a few persons who behaved in a similar manner. This would indicate that, although they had a certain amount of antitoxin in the blood, they were not in a "sensitized" state.

This group revealed the same individual variability in the amount of antitoxin produced and lost in the course of active immunization as other groups reported by us. Two subjects dropped below 0.1 unit when tested ninety days after the second dose. Four individuals dropped below this level six months after the second dose, while two subjects maintained it for a little over nine months. Case 6611 showed 2.0 units of antitoxin when tested six hundred and seventy-nine days after the second dose. Case 6617 had 0.1 unit six hundred and eighty-seven days after the second injection.

Another group of 11 adults (group B, Table II) received the same treatment as group A, but the second dose was given seven hundred and thirty days after the first injection. In this series, we attempted to determine the rapidity of development of the protective titer of 0.1 unit, as well as its duration following the injection of the second dose of tetanus toxoid.

Seven subjects tested on the third day after the second dose showed –0.1 unit. Two persons tested on the fifth day also showed –0.1 unit. One of these when retested two days later showed +0.10 but –0.25 unit. Of three cases tested on the sixth day, two were found to be below the protective titer, while the third one had 0.25 unit. Two subjects still had –0.10 unit when tested on the seventh and ninth days, respectively. The antitoxin titer of the entire group went up during the second week after the administration of the second dose of toxoid, so that by the fourteenth day they all showed +0.25 unit. Two subjects dropped below the protective level of 0.10 unit three and and six months after the second dose, respectively. There was a gradual loss of antitoxin in the remainder of the group but all, with the exception of Case 6683, still showed +0.10 when tested nineteen months after the second injection of toxoid.

In view of these findings it is apparent that individuals undergoing active immunization against tetanus must be protected by the administration of a prophylactic dose of serum if injured during the interval of time that elapses between the injection of the two doses of toxoid, and for a week or two following the second dose, when the protective antitoxic immunity has not as yet fully developed.

In reference to the effect of the "injection de rappel" of the French, Bergey and Etris[11] administered a third dose of 1 c.c. of alum toxoid to 3 persons, two years after the second injection. Thirty days later, the content of antitoxin in their serums was 2.0, 2.0, and 1.5 units per cubic centimeter, respectively. The authors felt that the antitoxin content of these patients' blood had been raised to a point where they were protected for another period of two or three years.

Hall[6] administered a third dose of alum toxoid to a group of 13 adults seven and one-half months after the second injection. There was no change in their titer when tested two days after the repeat injection, but when tested

TABLE III

GROUP C

ANTITOXIN RESPONSE AFTER THE INJECTION OF A THIRD DOSE OF TETANUS TOXOID, ALUM PRECIPITATED, REFINED

Titer expressed in units of tetanus antitoxin per cubic centimeter of blood serum.

CASE	NO. OF DAYS BETWEEN SECOND AND THIRD DOSES	CONTROL TITER	THIRD DOSE NO. 5969-1	DAYS AFTER THE THIRD DOSE TITER												
				4	7	9	14	28	33	92	139	182	212	273	360	
6642	308	+0.01 −0.10	0.5 c.c.		0.10		+0.25	+0.25		+5.0		+0.25		+0.10 −0.25	−0.10	
6644	308	+0.01 −0.10	0.5 c.c.		0.25		+0.25	+0.25		+0.25		+0.50 −0.50		0.50	0.25	
6645	308	+0.01 −0.10	0.5 c.c.		+0.10		+0.25	+0.25		+0.25		+0.10 −0.25		0.10	0.10	
6714	230	+0.01 −0.10	0.5 c.c.		+0.25		+0.25	+0.25		0.10	0.10			+0.10 −0.25		
6614	308	−0.10	1.0 c.c.		−0.10		+0.25	0.25		−0.10		0.01		0.01	0.01	
6649	308	+0.01 −0.10	1.0 c.c.		0.25		+0.25	+0.25		+0.25		0.10		+0.01 −0.10	−0.10	
6657	308	+0.01 −0.10	1.0 c.c.			+0.25		+0.25								
6643	200	0.01	0.5 c.c. No. 76812-1	+0.01 −0.10	+0.10 −0.25	+0.25			0.25		0.10	−0.10	+0.01 −0.10			

again on the seventh day, there was noted a very marked rise in the antitoxin content of their blood, reaching from 1 to 25 to 50 units. From an average control titer of 0.032 unit, there was an increase to an average titer of 9.6 units of tetanus antitoxin.

Cowles[8] administered a third dose of alum toxoid to 10 subjects, thirty-nine weeks after the second injection. No increase in titer was noted until the fourth day after the repeat injection, when 1 out of 6 subjects tested showed 0.1 unit. On the fifth day, 4 subjects had +0.1 unit, while 3 still showed 0.05 unit or less. On the seventh day, 3 cases had 0.50 unit, 1 showed 5 units, and 1 still showed 0.05 unit. The latter when tested twenty-four hours later showed a titer of 0.20 unit. On the tenth day, 1 subject showed 0.50 unit while another one had 10 units. The latter on follow-up tests revealed a decided drop in the antitoxin content of his blood, showing 5 units on the fortieth day and 2 units one hundred and forty days after the repeat injection. Cowles concluded that the response to a subsequent injection is not significant until the fourth or fifth day to prevent tetanus, but did not know whether or not this was soon enough. Although the influence of such a delayed rise in titer cannot be determined without further study, Cowles felt that upon injury, the repeat injection of toxoid should certainly be made if antitoxin is to be withheld.

Seven subjects (group C, Table III), members of our groups A and B, received a third dose of tetanus alum-precipitated toxoid No. 5969-1, two hundred to three hundred and eight days after the second injection. This lot of toxoid was freshly prepared from a toxin containing over 20,000 M.L.D. The injection of 0.5 c.c. of this toxoid into a group of standard guinea pigs produced 5.0 units of tetanus antitoxin per cubic centimeter of blood serum at the end of six weeks. Similar values were produced following the injection of 1.0 c.c. of this antigen. The eighth member of group C was injected with the aged toxoid No. 76812-1, used for the basic course of immunization. Their serums showed –0.1 unit before injection of the repeat dose. All but three subjects got 0.5 c.c. of the toxoid; the rest received 1.0 c.c.

Case 6643, whose control titer was 0.01 unit, showed +0.01 –0.10 unit when tested four days after the third dose. On the seventh day, all patients but one showed 0.1 unit or more. The exception when tested again a week later showed +0.25 unit. Case 6614 dropped to –0.10 unit when tested three months after the repeat injection. The rest still showed good protective values. Six months after the third dose another subject dropped below 0.1 unit. Three months later, a third patient showed –0.1 unit. When tested one year after the repeat injection, over half of the members of the group had dropped below the minimum protective level of antitoxin.

Upon comparison of the type of response obtained after the third dose of toxoid with that obtained following the injection of the second dose, one finds that in four persons, the third dose was followed by a more prolonged maintenance of the protective titer. In two, the duration of the protection was identical. As in the case of the second dose, the response following the injection of a repeat dose of toxoid is variable and in some subjects may be short-lived. Hence a false sense of security may result. A definite period of time elapses

before a protective titer develops following the repeat injection. In some cases, it may be longer than a week. The question naturally arises as to whether tetanus infection may not develop before the antitoxin titer has a chance to reach a protective level. Additional data should be collected before a definite conclusion can be reached on this point. Meanwhile, it is important to insist on conservative standards of protection.

It may be that antitoxin per se is not the sole factor concerned in the process of immunity against tetanus. Both Sneath and Cowles present suggestive experimental evidence in support of this view. In an addendum to his report, Cowles cites the case of a subject who had recovered from tetanus eight years before the injection of 0.5 c.c. of alum toxoid, and who reacted to this first injection in the same manner as normal individuals do. This would indicate that the toxin concerned in the attack of tetanus had not, in this case, constituted an antigenic stimulus, since a state of "sensitization" did not result.

Similarly Case 6645 of our group A had also recovered from tetanus in 1923. Ten years later his antitoxin titer was found to be –0.003 unit. There was no appreciable increase in the antitoxin content of his blood following the first injection of alum toxoid, indicating that the tetanus infection from which he had recovered had not affected his immunity mechanism in the same fashion as the injection of the first dose of toxoid. The response of this subject to subsequent injections of toxoid was similar to that of normal individuals.

Case 6643 received a fourth dose of alum toxoid (0.5 c.c.) two hundred and twelve days after the third injection, when the antitoxin content of his blood was found to be +0.01 –0.10 unit. Seven days later, his titer was +0.25 unit. When tested again four months later, he showed 0.25 unit. One year after the injection of the fourth dose of toxoid, the antitoxin content of his blood was found to be +0.1 unit.

COMMENT

The most important question requiring further investigation is the determination of the actual amount of antitoxin necessary to protect an individual against tetanus. More experimental data and perhaps field work, possibly in war zones, rigidly controlled to satisfy statistical requirements are needed. Around it hinge the limitations of active immunization against tetanus as brought out by this study. At present active immunization may prove to be of value in military service and in certain phases of civil life where injuries occur repeatedly. It will also prevent the occurrence of "delayed" or so-called "chronic tetanus."

CONCLUSIONS

1. A protective antitoxin titer of 0.1 unit or more develops in five to fourteen days following the injection of a second dose of alum toxoid, even when the latter is given two years after the first dose.

2. The duration of the protective level of antitoxin varies a great deal. It may disappear within ninety days after the second injection or it may last over two years.

3. The antitoxin titer can be raised to a protective level by the injection of a third or subsequent doses of alum toxoid.

4. From five to more than seven days elapse after the "repeat" injection of toxoid before there appears 0.1 unit of antitoxin in the blood of actively immunized persons.

5. The immunity that develops following the injection of a "repeat" dose of alum toxoid is also variable in duration. It may drop below 0.1 unit in three to six months after the third or subsequent injections.

6. If an injury occurs during the interval of time that elapses before the basic course of immunization is completed and within a few days after the second or subsequent injections of alum toxoid when the antitoxin level of the blood has not as yet reached 0.1 unit, it may be necessary to resort to passive immunization in order to insure full protection against tetanus.

The antitoxin titrations reported in this paper were carried out at the Mulford Biological Laboratories. Sharp and Dohme who kindly supplied me with the necessary material for immunization. I also wish to thank the Sackville Mills Co., Wallingford, Pa., for their cooperation in securing their personnel to serve as subjects for this investigation.

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ELECTROCARDIOGRAPHIC CHANGES FOLLOWING THE INTRAVENOUS ADMINISTRATION OF MAGNESIUM SULFATE

An Experimental Study on Dogs*

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THE present study was undertaken to determine the effect upon the conductive system, as measured by the electrocardiogram, of disturbances in the chemical balance ordinarily maintained by the body. Alteration in the magnesium content constituted the first portion of this study. Sudden death following the injection of a magnesium salt for treatment of epilepsy and other conditions is not an uncommon occurrence. The mechanism of such untoward results, however, has not been adequately described.

Jolyet and Cahours[1] showed that the intravenous injection of a solution of magnesium salts caused loss of motility of voluntary muscles, and Matthew Hay[2] demonstrated that respirations ceased following large doses, although the heart continued to beat slowly. Larger doses produced a sudden cardiac standstill during diastole. Matthews and Brooks,[3] in a series of experiments on dogs, concluded that the action of magnesium sulfate upon the heart is essentially a depression of the cardiac nervous mechanism, resulting in loss of tone, but that it retains its irritability and that a sufficiently strong stimulus will result in contraction.

Tedesco[4] made observations of the effect of magnesium chloride upon the electrocardiogram. He concluded that it produced a positive chronotropic effect on the action of the heart, and that the speed of propagation of the stimulus from the auricle to the ventricle was appreciably diminished. He was unable to draw conclusions concerning its effect upon the bundle of His, but was of the opinion that magnesium had a stimulating effect upon the electrical condition of the ventricles.

Zwillinger[5] observed the effect in man of the intravenous and intracardiac injection of magnesium sulfate when arrhythmias were present. He concluded that it plays a rôle in combating certain forms of paroxysmal tachycardia and extrasystoles. He was able to show experimentally that magnesium sulfate had a beneficial effect in combating digitalis intoxication.

METHODS

In all experiments the dog was placed on the left side. No anesthetic was used. Electrodes were attached to the right front and left rear legs, thus corresponding to the standard second lead. Electrocardiograms were made on

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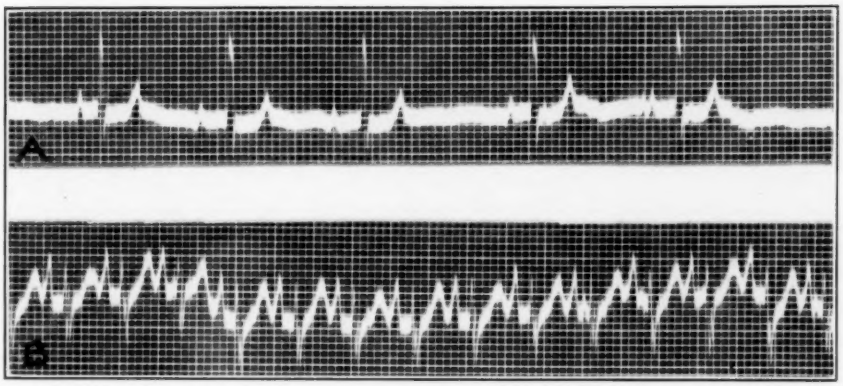

Fig. 1.—*A*, Dog 2. Normal graph, rate 75. *B*, Following the injection of 4 c.c. of a 20 per cent $MgSO_4$ solution. Note increase in rate (190).

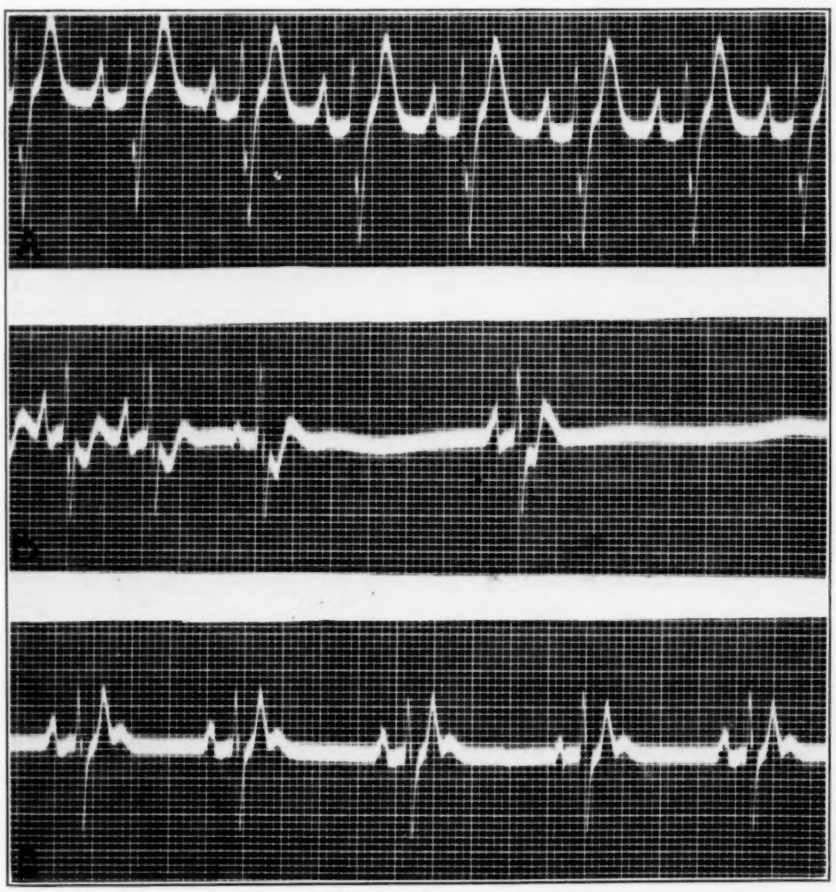

Fig. 2.—*A*, Dog 2. Continuation from Fig. 1. This graph was taken following injection of 22 c.c. of solution. Note increase in the amplitude of all complexes. Rate 110. Respirations have now ceased. P-R and QRS intervals increased 0.06 second. *B*, Two minutes later with cessation of activity. *C*, Short resumption of activity after one minute, lasting only thirty seconds.

a string galvanometer type machine. The magnesium salt was injected into the right saphenous vein. Electrocardiograms were made during injection and at varying intervals from two minutes to two days, as described hereafter.

RESULTS

Experiment 1.—Dog 1, weight 10 kg.; Dog 2, weight 13.1 kg. Following injection of 10 c.c. of 20 per cent magnesium sulfate solution, there was an acceleration of rate from 75 to 90. This coincided with a marked though transient increase in respiration. Otherwise the only other remarkable change was an increased excursion of the T-wave.

The solution was then injected at the rate of about 2 c.c. per minute in Dog 1, and 1 c.c. per minute in Dog 2, and graphs taken at two-minute intervals.

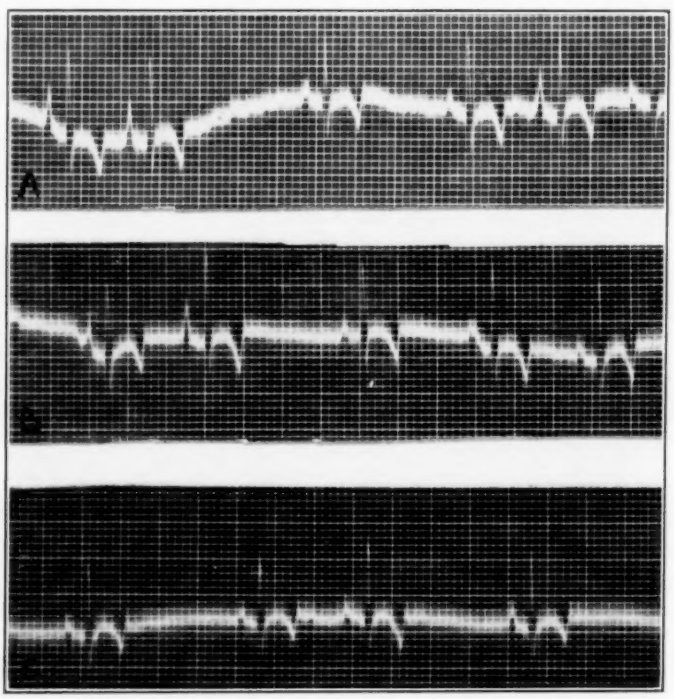

Fig. 3.—*A*, Dog 3. Normal graph. *B*, Ten minutes after injection of "therapeutic dose" of $MgSO_4$, P-R interval prolonged 0.02 second. *C*, One hour later. Return to normal.

The rate increased markedly until in each instance 18 c.c. had been injected. Thereafter it fell slowly. At 28 c.c. respirations ceased, and the heart rate had fallen to 70. Two minutes later the heart suddenly stopped contracting.

As the rate increased (Figs. 1 and 2) there was also an increase in the amplitude of all complexes with a prolongation of both auricular and ventricular conduction time. After a short period of inactivity, the heart resumed a sinus rhythm, although there was marked distortion of the various complexes. In Dog 1, after a second period of complete inactivity, the heart was reinactivated following injection of calcium gluconate, and when cessation of activity again occurred, artificial respiration was followed by a rhythm of ventricular origin which lasted for a short period.

Experiment 2.—Dog 3, weight 9 kg. In this animal the effect of an intravenous injection of magnesium sulfate, in dosage proportionate to the amount sometimes given therapeutically, was noted (22 mg. per kg.). Five minutes after administration, the rate had fallen from 100 to 80, the P-R interval was increased (0.02 second), and the T-wave, originally negative, became more so by 25 per cent. There was no other discernible difference. These changes were present ten minutes after injection but had entirely disappeared at the end of an hour (Fig. 3).

A graph made twenty-four hours later was identical with one made at the beginning of the experiment.

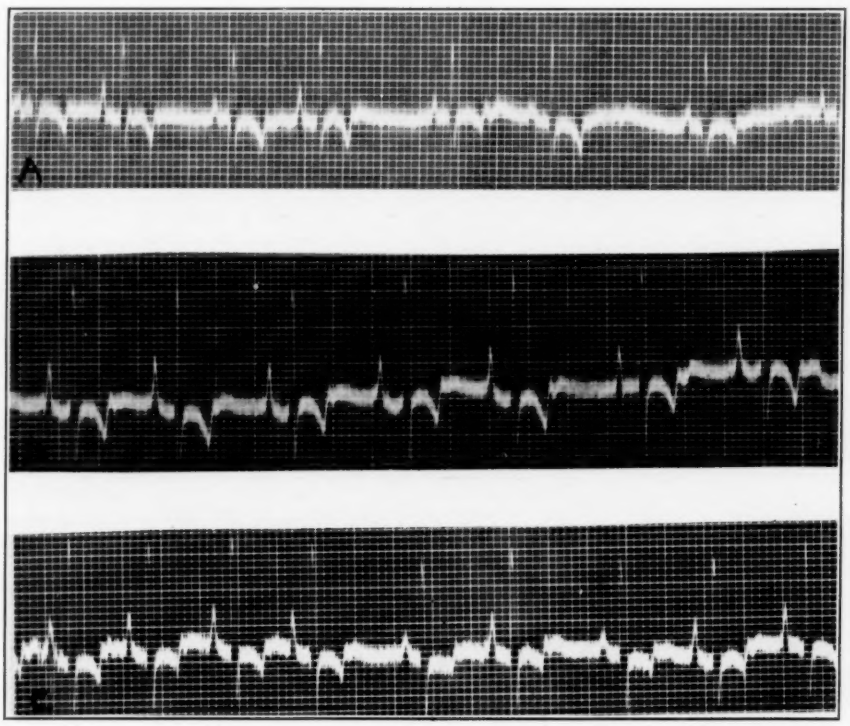

Fig. 4.—*A*, Dog 3. Normal graph. This graph was taken twenty-four hours after those in Fig. 3. *B*, thirty-five minutes after injection of 9 c.c. of a 20 per cent $MgSO_4$ solution in divided doses. Note increased amplitude of all complexes with prolongation of both auricular and ventricular conduction time. *C*, Eighty-five minutes later. Some effect still evident.

Experiment 3 was carried out essentially as Experiment 1, except after injection of 9 c.c., of 20 per cent solution of magnesium sulfate, at which point pronounced changes had occurred, the administration of the drug was stopped and the dog allowed to recover. Increased excursion of the P- and R-waves as well as an increased auriculoventricular conduction time persisted at the end of eighty-five minutes, indicating that in such doses the effect is not a transient one (Fig. 4).

Experiment 4.—As magnesium sulfate had been used in the preceding procedures it was decided to use another salt of magnesium to determine whether the latter substance was solely responsible for the effects noted.

Consequently magnesium chloride was given in an amount containing an equivalent weight of magnesium as employed in the first experiment. The findings were essentially the same as resulted from the use of the sulfate.

CONCLUSIONS

Electrocardiographic studies indicate that magnesium, when injected intravenously, has a definite effect upon the cardiac conductive system.

This effect consists of an early acceleration, followed by a slowing with delay in auriculoventricular and ventricular conduction time and increased excursion of all complexes. This effect may persist for more than an hour after injection of the salt, but is not permanent.

Respirations cease before cardiac activity stops. When the latter occurs, it does so suddenly with a preceding bradycardia, but without any arrhythmia. The heart may spontaneously begin to beat, even after complete cessation of activity.

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THE INCIDENCE OF POSITIVE IMMUNOLOGIC REACTIONS FOR UNDULANT FEVER*

Studies of 5,000 Blood Serum and 491 Skin Tests

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WITHIN the past ten years the medical profession has become increasingly aware of undulant fever, a disease formerly considered as uncommon or rare. A larger number of acute cases of undulant fever are now being recognized because physicians are considering the possibility of the clinical entity in febrile conditions which are not otherwise explained by the physical findings.

It is apparent, however, that few physicians realize the great importance and the high incidence of the chronic and ambulatory forms of the disease which so often cause only vague aches and indefinite complaints. In the past, and even now, patients with these indefinite symptoms have often been dismissed with a presumptive diagnosis of "chronic fatigue" or "neurasthenia."

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The final diagnosis of undulant fever in both the acute and chronic forms depends on laboratory data and includes bacteriologic and immunologic tests. In the chronic form particularly, the symptoms and physical findings are so variable as to make very questionable a diagnosis on a purely clinical basis. Due to difficulty in isolating the causative organism in chronic cases, immunologic procedures, such as the agglutination test and the intradermal test, must be employed to make a definite diagnosis.

Epidemiologic studies, based on laboratory procedures, have been undertaken in various parts of the world to determine the incidence of human brucella infections. The results have been astounding because of the large number of cases, particularly of the chronic type, which have been discovered. The literature on undulant fever is voluminous and references will be found in many of the articles on the subject.

This report covers work which has been carried out to determine the incidence of positive immunologic evidences of undulant fever in unselected patients admitted to a general hospital and its out-patient department.

In this study 5,000 consecutive specimens of blood submitted for the Wassermann test by the hospital and its out-patient department were examined for brucella agglutinins by the macroscopic tube method. The antigen used for the agglutination test was made from a strain of *Br. melitensis* var. *abortus*, recovered from a human case of acute undulant fever. The organisms were grown on a 2 per cent nutrient agar medium for forty-eight hours, and then suspended in normal saline solution containing 0.5 per cent phenol. The suspension was kept in the icebox for forty-eight hours and at the end of this time proved to be sterile. This was diluted to a turbidity of 300 (silica standard) with 0.5 per cent phenolized normal saline solution. For the test the serum was placed in small test tubes and antigen was added to make two final dilutions of the serum: 1:25 and 1:100. The tubes were incubated in a water bath at 54° C. for four hours, then placed in the icebox overnight and observed the following morning. They were returned to the icebox for twenty-four hours longer and the readings made again.

Of the 5,000 blood serums examined routinely, 51 showed an agglutination titer of 1:25 and 9 had a positive agglutination reaction in both the 1:25 and 1:100 dilutions. In all, 60 patients had positive agglutination reactions. Follow-up studies revealed that in 39 of this group on whom positive reactions had been obtained, 24 (62 per cent) were then living on farms. Thirty-one (79 per cent) of the 39 stated that they had drunk mostly raw milk in the past two years, while only eight (21 per cent) drank little or no raw milk during this time.

In this series of 5,000 blood serums, 1,900 (38 per cent) were from men and 3,100 (62 per cent) were from women. Of the 60 positive serums, 21 (35 per cent) were from men and 39 (65 per cent) were from women. This makes an almost equal incidence of positive agglutination reactions among the men and women of this series.

At the time the agglutination tests were being carried out, intradermal tests were also done on the hospital patients (except those seriously ill and

infants less than six months of age). A definite majority of these hospital patients had been living on farms or in farming districts.

The antigen used for the intradermal tests was the same as that for the agglutination tests, except that it was diluted ten times with sterile saline solution containing 0.5 per cent phenol, then heated in a water bath at 60° C. for one hour, and tested for sterility. One-tenth cubic centimeter of this antigen was injected intradermally in the ventral surface of the forearm of the patients.

Local reactions, which occurred during the first twenty-four hours after injection, were disregarded. Mild general reactions occurred in some of the patients in twenty-four to thirty hours. These later showed positive intradermal reactions. In the positive reactors there developed at the site of the injection, in about forty-eight hours, a tender edematous area with central induration, varying from 1 to 2 cm. in diameter, and a peripheral zone of erythema, varying from 2 to 8 cm. in diameter, depending on the severity of the reaction. Erythema alone, without induration, was regarded as a negative reaction. Table I shows the basis for grading the reactions, which was the diameter of the area of induration and of erythema.

TABLE I

GRADING OF SKIN REACTIONS

INTERPRETATION	GRADE OF REACTION	DIAMETER OF INDURATION (CM.)	DIAMETER OF ERYTHEMA (CM.)
Negative	0	0	0
	1	0	1 or more
Positive	2	1	3
	3	1 to 2	4 or more without ulceration
	4	1 to 2	4 or more, with ulceration (in seven to ten days)

In some of the more severe reactions there were reddish streaks extending a short distance up the forearm, suggestive of a lymphangitis. The reaction was usually at its height in from seventy-two to ninety-six hours, and gradually subsided during a period of a few weeks to three months. In some of the more severe reactions, at the end of a week to ten days, the central area softened and then ulcerated with the discharge of thick leucocytic material which did not show bacteria in either smears or cultures.

TABLE II

RESULTS OF INTRADERMAL TESTS ON 491 HOSPITAL PATIENTS

HOSPITAL WARD	NUMBER OF TESTS DONE	NUMBER OF POSITIVE REACTORS	PERCENTAGE OF POSITIVE REACTIONS
Men's Medicine	90	18	20.0
Men's Surgery	83	7	8.4
Total Men	173	25	14.4
Women's Medicine	78	11	14.1
Women's Surgery	91	9	9.9
Obstetrical	87	12	13.8
Total Women	256	32	12.5
Children's	62	3	4.8
Grand Total	491	60	12.2

Table II shows the results of 491 intradermal tests made on patients in the different hospital wards. The patients in the men's medical ward yielded the highest per cent of positive intradermal reactions, while the lowest per cent occurred among the pediatric patients.

A positive agglutination reaction for undulant fever is generally considered as indicating either present or past infection with brucella organisms. The intradermal reaction is believed to have the same significance, but it often is present in the absence of an agglutination reaction. In this series, no patients were found who gave a positive agglutination reaction and a negative intradermal reaction. However, many patients with a negative agglutination reaction gave a positive intradermal reaction. The intradermal test yielded a much higher per cent of positive reactions (12.2) than did the agglutination test (1.2).

Authorities are well agreed that the ingestion of raw milk is a common source of undulant fever. It is interesting to note that of the 60 patients who had positive skin reactions, 55 (91.7 per cent) gave a history of drinking mostly raw cow's milk; one (1.7 per cent) drank raw goat's milk; two (3.3 per cent) stated that they drank only pasteurized milk; and two (3.3 per cent) consumed only canned milk during the past two years. Of the 39 patients showing positive agglutination reactions, on whom a milk history was obtained, 31 (72 per cent) drank mostly raw milk during the past two years. It is apparent from these figures that there seems to be a definite direct relationship between the use of raw milk and the occurrence of positive agglutination and skin reactions for undulant fever.

CONCLUSIONS

Five thousand samples of blood have been examined and 491 intradermal tests have been carried out to determine the incidence of positive immunologic reactions for undulant fever.

Sixty (1.2 per cent) of the 5,000 blood samples yielded positive agglutination reactions. Sixty (12.2 per cent) of 491 patients showed positive intradermal reactions.

The incidence of positive immunologic reactions is approximately the same for the men and women in this series.

Patients on the men's medical ward showed the highest incidence of positive intradermal reactions, while the children in this series had the lowest.

There seems to be a direct relationship between the occurrence of positive immunologic reactions and the ingestion of raw milk.

Brucella infections appear to be relatively common, as evidenced by immunologic tests carried out on patients admitted to a general hospital and its outpatient department.

THE CLINICAL UTILIZATION OF BLOOD STUDIES*

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IT IS common knowledge that the practice of medicine is not a fixed but a changing art. The concepts of today are so continually modified by the studies of tomorrow that the theories of today are sometimes the facts of tomorrow, just as the facts of yesterday may become the archaisms of today.

It is this continual realignment of facts, together with the equally constant readjustment of fact and theory, which forces the conscientious physician to continued study.

Perhaps among the more startling, and at times even disconcerting, phases of early days in practice is an appreciation of the dearth of the typical text-book case. In contrast to the clear-cut, definite entities of the lecture hall and clinic, one seems to see rather diagnostic problems bristling with complexities, each of which constitutes in essence an examination of one's knowledge in its entirety. These experiences, if utilized and digested, lead inevitably to the realization that the physician must acquire and develop an investigative viewpoint. It is not enough to give the disease a name. Its nature, its causative factor or factors, and the mechanism whereby the effects leading us to suspect its presence are manifested must all be appreciated and understood if attempts to control it are to be intelligently conceived and wisely directed.

To this end, fortunately, the physician of today may call to his aid innumerable refinements applicable to the examination of the patient, among them the numerous resources of the clinical laboratory.

So many and so great have been the advances in the field of laboratory medicine that there has been at times some indication of unfortunate tendency to utilize them to the neglect of the six most valuable assets in the study of any patient or any disease, to wit: one's five senses, carefully and thoroughly trained for the work at hand, and, perhaps most valuable of all—common sense in their utilization and direction.

The laboratory is not a diagnostic slot machine. It constitutes primarily and essentially simply *a phase in the examination of the patient.* It must be used as an adjunct to the ordinary methods employed in the study of the patient and disease and as such cannot safely nor profitably be neglected. And just as the clinical pathologist must be a physician with sufficient clinical training to enable him to interpret correctly the data accruing from his examinations, so the physician must be sufficiently a clinical pathologist to select from the multitude of those available the particular procedures most likely to be informative in the particular problem at hand.

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Just as Lord Moynihan is said to have regarded every surgical operation as an experiment, and Sir William Jenner to have looked upon the administration of a drug as a research problem, so the physician must develop the investigative viewpoint in the study of his patient, utilizing to that end as many and as varied avenues of approach and study as the diagnostic and therapeutic problem in that case may necessitate.

Having thus by these broad generalizations prepared the way for a more particular discussion, we may now discuss as briefly as may be the utilization and significance of various blood examinations in the study of disease. In so doing, we shall not select the unusual nor the ultrascientific, but rather the more common and homely blood studies which, perhaps because they are familiar, are not always appreciated or utilized to the fullest extent.

THE BLOOD COUNT

The blood count coupled with the cytologic study of the stained smear is obviously of paramount importance in the study of primary disorders of the blood-forming organs. But primary disorders of the hematopoietic system are rare, while disturbances of the blood as a concomitant or aftermath of disease in general are so common that it can be said with confidence that there is hardly any disease in which a systematic hematologic study will not prove of material aid and value.

As everyone knows, an accurate diagnosis of anemia is impossible without a blood count, for symptoms cannot be relied upon. Pallor of the skin is entirely unreliable because it depends, not only upon the amount of hemoglobin per unit volume of blood, but also upon such varied factors as the skin thickness, the amount of skin pigment, the presence or absence of edema, the state of contraction or relaxation of the capillaries, and the degree to which the individual is exposed to or protected from ultraviolet radiation.

In like fashion other symptoms associated with anemia, such as weakness, shortness of breath and edema, may be present in many other conditions and absent in the presence of anemia.

To recognize an anemia, therefore, and especially to recognize its type and, by inferential deduction, the underlying responsible mechanism—an understanding of which is essential to intelligent treatment—recourse must be had to examination of the blood.

Reliance upon hemoglobin determinations alone, as is sometimes done, is not sufficient and may, indeed, prove misleading; a simultaneous study of the red cells is essential.

It is now clear that the production of anemia depends upon two fundamental factors: an increased rate of loss or destruction of red cells, or a decreased rate of formation, either of which may be brought about by a variety of causes.

From the standpoint of efficient treatment the necessity of determining not only the presence of an anemia but its type is apparent. Fortunately, this can be determined by a careful study of the characteristics of the red blood cells, so that, from this angle, the most important feature of the blood count is not the

amount of hemoglobin and the number of erythrocytes, but that portion of the report describing what is seen in the stained smear.

We now divide anemias into three main groups: normocytic, macrocytic, and microcytic, in accordance with the size of the erythrocytes: normal, larger, or smaller than normal.

This is of therapeutic as well as academic importance. For all macrocytic anemias depend upon a deficient supply to the bone marrow of the antipernicious anemia principle, and hence respond to the administration of this principle. Microcytic anemias, in which the cells are hypochromic as well as smaller than normal, are largely dependent upon an insufficient supply of the substances essential to hemoglobin formation, of which iron is the most important. Hypochromic microcytic anemias may thus be expected to respond to iron therapy. Macrocytic anemias, on the other hand, can be produced in a variety of ways, and their etiology must be determined before intelligent therapy can be planned.

Important and valuable as are these simple procedures, there are others of ancillary and sometimes great importance in the study of a particular problem. Among these are the various indices of color, volume, and saturation.

The color index expresses the mean hemoglobin content of a single cell as compared with the content of a normal cell. The volume index expresses the ratio between the average size of the red cells in the blood examined and the average size of the red cell in a normal blood. The saturation index expresses the relation of the mean corpuscular hemoglobin concentration to normal and hence indicates the relation of the hemoglobin concentration to cell size.

Under normal conditions all three indices range between 0.8 to 1.2 and hence will be found within this range in normocytic anemias which are not characterized by general changes in the size of the red cells.

In the macrocytic anemias, where the erythrocytes are large, the volume and color indices will naturally exceed normal, while the saturation index remains within the normal range or shows but a slight increase.

In the hypochromic microcytic anemias all the indices, as can be foretold by the type of anemia, are below the normal range.

If blood studies are thus seen to be important and essential for the accurate diagnosis of anemia, and of equal importance at times in indicating the type of therapy most likely to be efficient, they are also of great value as a measure of control of the results of therapy.

In the icterus index, assuming absence of hepatic disease or biliary obstruction, we have a gauge of the degree of erythrocyte destruction; while the reticulocyte count furnishes an equally accurate index of the degree of red cell regeneration.

In the absence of hepatic or biliary disease an icterus index above 6 indicates increased red cell destruction, the index increasing as the erythrocyte destruction increases.

A reticulocyte count of less than 0.5 per cent is an index of abnormally slow regeneration; a count of 4 per cent or over in individuals over 4 years old indicates an increased rate of erythrogenesis, and this procedure may thus be utilized as a measure of therapeutic response. However, as the investigations

of various workers have clearly shown, the degree of reticulocyte response to therapy is governed not only by the activity of the therapeutic agent and the adequacy of the dose, but also by the initial level of the red cell count and can only be used as a therapeutic gauge when the initial red cell count is 3 million or less. Above this initial level reticulocyte counts are irregular and inconstant.

The reticulocyte count is also of some value in differential diagnosis, being increased in sickle-cell anemia, acute febrile hemolytic anemia (Lederer's anemia), familiar hemolytic icterus, myelogenous anemia, lead poisoning, malaria, and any anemia characterized by an increased rate of blood destruction.

A marked, but transient increase in the reticulocyte count follows acute hemorrhage and, occurring in pernicious anemia, forecasts the approach of a spontaneous remission.

Even when a reticulocyte count is not done a definite, but by no means as accurate, idea of the presence or absence of regenerative activity and a rough approximation of its degree may be had from a study of the stained smear with particular reference to the presence, absence, or incidence of polychromatophilia (of which basic stippling is one manifestation) and nucleated red cells, all of which represent phases of erythrocytic formation.

It is, of course, unnecessary to dwell upon the vital importance of the blood count—in which is included the study of the stained smear—in the diagnosis and study of diseases of the blood itself, such as polycythemia vera and the leucemias.

Blood Count in Concealed Hemorrhage.—The blood count is not infrequently used as a means for the detection and measurement of concealed hemorrhage, but not always used to the best advantages. For example, a patient may present signs and symptoms suggesting, but not clearly, the possibility of some abdominal catastrophe accompanied by hemorrhage, such as a ruptured ectopic or a laceration of an organ.

Not infrequently, information for or against such a diagnostic possibility is sought by means of repeated serial blood counts or by repeated determinations of hemoglobin and red cell counts. Such use does not always take into account the sequence of events following hemorrhage.

In the case of relatively small hemorrhages the effect upon the hemoglobin and red cell count may be so minor as to be unimportant and inconclusive until, as a result of long-continued demand, evidence appears of fatigue on the part of the hematopoietic system, and this, of course, is of little use where the emergency is acute.

In the case of relatively large hemorrhages, no change will be seen in hemoglobin determinations or red cell counts until some hours—and sometimes quite a few hours—later when both will drop as a result of dilution of the blood by the fluids withdrawn from the tissues to compensate for the fluid lost in the hemorrhage. Here again, the effect may appear too late to be of aid in an acute emergency, such as those just cited.

In such case, the important information of clinical use and value is to be obtained, not from a study of the hemoglobin and red cells, but from the total and differential counts. For hemorrhage into a closed cavity lined with an absorbing membrane such as the peritoneum produces an early and marked

polymorphonuclear leucocytosis, and this finding, showing a progressive leucocytosis, may be used with confidence as a sign of concealed hemorrhage in such localities.

Blood Count in Infections.—While many infectious diseases are capable of producing more or less anemia, the most characteristic, most definite, and most diagnostically valuable changes are seen in the alterations produced in the number and characteristics of the leucocytes and this discussion will therefore be confined to these.

It is now many years since Sondern first enunciated the now commonly accepted rules that the severity of an acute infectious process was in large measure suggested by the percentage of polymorphonuclear leucocytes in the differential count, and the reacting ability of the patient in large measure suggested by the total leucocyte count.

Oddly enough, these two axioms still suffice for some to the utter neglect of the many and important refinements now associated with the study of the leucocytes in the presence of infection.

While the dicta just mentioned are in large measure true, they cannot be blindly relied upon for the following rather obvious reasons:

In the first place leucocytosis and leucopenia are primarily manifestations of reaction to stimuli, just as, indeed, are all the varied manifestations of disease which we call symptoms.

There are, however, several variable factors involved in the production of these phenomena. One is the nature and intensity of the stimulus; another the ability of the patient to respond to it. Obviously, therefore, alterations in the total and differential leucocyte count will depend, not only on the presence of infection but also, and to no small extent, upon the ability of the patient to respond to the stimuli exerted by the phenomena attendant upon the pathologic processes resulting from the disease.

A diffuse and spreading infectious process may be expected to be accompanied by leucocytosis, both total and polymorphonuclear, unless the toxic absorption is so massive or the infection so virulent that the patient is overwhelmed. In such a case the total leucocyte count may approximate normal, or there may even be a leucopenia.

On the other hand, a severe and virulent infectious process may be so located and so well walled off that absorption from it, and hence stimulation, may be reduced to a minimum, and the leucocytic response correspondingly less marked.

Thus it is that, as is always the case in any method of examination of the patient, among which are laboratory procedures, the import and value of the procedure depends, not upon its results, but upon their interpretation.

It would not be safe, for example, to base a diagnosis of acute lymphatic leucemia upon a leucocytosis of 100,000 and a lymphocytosis of 98 per cent in a child, because such a picture may be encountered as an evidence of reaction to pertussis vaccine. Similarly, a diagnosis of myelogenous leucemia cannot be safely based upon a high total count and the presence of myeloblasts, for such findings may occur as what has been termed the "leucemoid reaction" to infection.

Failure to obtain from the leucocyte counts the utmost in clinically useful information arises from various causes, among which may be mentioned, the paying of too much attention to the cells which are increased and not enough to those which are decreased.

An old but pertinent observation, often forgotten nowadays, relates to the effect of infections upon the eosinophiles. A decrease in the eosinophile count in conjunction with an increased polymorphonuclear percentage was recognized as indicative of a pyogenic process as long ago as 1902. This was the so-called "septic factor" which has definite value, so much so that a return of the eosinophile has a definitely favorable import.

The next step in the study of the leucocytic response to infection was the endeavor of various investigators to reduce the leucocytic reactions to formulas, resulting in various graphic reports, such as the Gibson chart, Walker's index of body resistance, the leucocyte index of Krebs, and so on. All of these had more or less value when properly computed and interpreted, but have now largely been discarded in favor of methods based upon the pioneer work of Arneth and its modifications by Schilling and others, so that the leucocytic hematology of infections is now largely dominated by the hemogram depicting the presence or absence of "shifts."

At first glance, such formulas appear somewhat complex, particularly as presented by some workers. In the ultimate analysis, however, they are based upon relatively simple principles.

It is apparent that the mobilization of leucocytes in response to infectious stimuli must necessitate the entrance of these cells from the various depots in which they are formed and stored. Where the stimulus is slight, the mobilization of mature cells may suffice. When this does not suffice, immature neutrophiles appear in the peripheral blood in proportion to the intensity and urgency of the demand.

Arneth was the first to call attention to the variations in the shape and character of the polymorphonuclear nucleus, and to associate these changes with the age of the cell in question, these investigations being later extended and revised by Schilling and others. All of these workers, in tabulating the various stages of nuclear morphology, naturally listed the youngest types first, followed by the other types in their supposed order of age and maturity.

When percentage estimations were made of these various immature forms, it was found that the proportion of younger forms was increased in proportion to the intensity of the reaction, and that hence the highest figures were seen under the youngest cells at the left of the form, and this finding was somewhat slangily expressed as "a shift to the left" to indicate a preponderance of immature leucocytes. From these relatively simple beginnings there has arisen an extensive hematologic structure with many complicated ramifications.

While there is no question as to the value of studies of leucocytic nuclear morphology in disease in general, and in infectious diseases in particular, there is some room for question as to whether or not the value of the procedure is enhanced by the complicated and sometimes even cumbersome nomenclature which has grown up about it.

It is for this reason that many attempts have been made to modify the methods of reporting such studies so that they may be made more simple and rapid in performance and especially more simply reported.

One of the simpler and yet very useful methods of determining the presence or absence of a shift, as well as its degree, is by dividing the total number of mature cells by the total number of immature cells found, the resulting figure being called the "nuclear index" (Boerner).

In the adult the normal index is 15 or over, an index of 10 to 15 denoting a very slight shift to the left; 5 to 10, a moderate shift to the left; and an index under 5, a marked shift.

The impossibility of elaborating leucocytic formulas applicable under all situations and of invariable significance is, of course, apparent. We must be prepared, then, for qualifications in the interpretation of variations in nuclear morphologic determinations, no matter how they may have been made.

For example, because of the extreme mobility of the bone marrow in infants and children, and the consequent even marked response to minor stimuli, such counts cannot be interpreted as in the adult, although an approximate basis for interpretation may be had by subtracting about 5 per cent from the immature neutrophilic percentage count in children 4 to 12 years of age, and about 10 per cent in infants under 4 years of age.

Likewise, these methods are inapplicable to noninfectious diseases, leucemias, pernicious anemia, and other myelophthisic anemias in which the presence of immature neutrophiles has no definite relation to prognosis. Nor can they be used in malaria because of the common occurrence of large numbers of young (staff) cells.

The interpretation of leucocytic variations may be summarized somewhat after this fashion.

1. A slight nuclear shift associated with a moderate neutrophilic leucocytosis and a persistence of eosinophiles is, when this picture is sustained, of favorable import.

2. A moderate nuclear shift in the presence of a slight leucocytosis, coupled with a decrease of eosinophiles and lymphocytes, warrants the diagnosis of infection, but is without prognostic significance.

3. A high leucocytosis associated with a marked shift, an absence of eosinophiles, and a decrease in lymphocytes, is of ominous import.

4. When, in the presence of lymphopenia, an absence of eosinophiles, and an increasing shift to the left, there is also a progressive fall in the total leucocyte count, the picture warrants a fatal prognosis with a considerable degree of certainty.

5. An eosinophilic leucopenia, when coupled with a rising total count, signifies a pyogenic infection; in the presence of a falling total count it suggests lack of resistance.

6. Lymphopenia coupled with a rising leucocytosis suggests a progressive inflammatory process; with a falling total count the prognosis is ominous.

7. Lymphocytosis associated with a return of the total count and nuclear index to normal is the usual accompaniment of convalescence.

Generalizations applicable particularly to variations in the nuclear morphology may be thus briefly summarized:

1. The peak of the immature cells is coincident with the height of the infection. As the disease process lessens and convalescence sets in, rod forms return to the normal level of 3 to 5 per cent. Their persistence at a high level suggests some complication.

2. In pneumonia a staff count of nearly 50 per cent of the total frequently accompanies the crisis. When the number of immature forms approaches 50 per cent in early pneumonia, the prognosis is ominous.

It will be noted that in all discussions of this matter of nuclear variation in the study of disease the bulk of the emphasis is laid upon the shift to the left. It should not be overlooked that the reverse of this, or a "shift to the right"—in other words, the return of mature leucocytes to the blood picture—is of some value as predicating the return to normal.

From even this cursory survey it is seen that while all these various refinements in the study of the leucocytes have greatly increased the prognostic significance of the leucocyte count, the procedure is still mainly a measure of activity and response to stimuli, with marked enhancement in differential diagnostic and prognostic significance.

So much emphasis has been laid upon the study of the nucleus that the value and significance of the study of the leucocyte as a whole has been somewhat overshadowed, except to the hematologist.

Changes in the cytoplasm and granules of the granuloeytic leucocytes have been so definitely found, so clearly described, and their significance so definitely indicated that the hematologist now recognizes the "toxic neutrophile" and utilizes it as a valuable prognostic indicator.

The changes denoting the toxic neutrophile are a tendency of the cytoplasm to take a bluer stain than normally, the presence of vacuoles in the cytoplasm, and variations in the size, number, and staining reaction of the granules, the toxic granule being larger, bluer, and not so numerous as in the normal cell.

Any one of these changes may be present alone or in combination, and, of course, may vary in degree. When present it is customary to grade their degree, as compared to the deviation from normal in the average cell of the patient's blood, by the arbitrary use of one- to four-plus.

As a rule, the presence of toxic neutrophiles is associated with a marked increase in the platelet count and their evaluation has been found to be of great prognostic value. When two of the changes indicative of the toxic neutrophile are present in three- to four-plus degree, a fatal outcome is almost inevitable.

The absence of toxic neutrophiles has not the reverse significance, however, as occasionally patients fail to develop such changes even up to the time of death.

Also, it is to be borne in mind that caution must be used in evaluating the significance of toxic changes in peritonitis and pneumonia, as variations of from three- to four-plus in any two of these changes indicating toxicity occur quite constantly in these two conditions with subsequent recovery.

Some workers calculate the percentage of neutrophiles showing toxic changes and report this as the degenerative index. It is practically as easy, however, and rather definitely more valuable, to estimate the degree of all the toxic changes noted and to base prognostic inferences upon this.

BLOOD SEDIMENTATION

Among the relatively recently developed procedures which has an accepted place in the field of laboratory studies is the sedimentation test.

Time does not permit discussion of the various theories advanced to explain its mechanism nor to mention the various technical factors which may produce anomalous and even fallacious results. It must be emphasized, however, that the blood sedimentation rate is a nonspecific phenomenon, entirely without specific diagnostic significance, and constituting but one of the varied manifestations of the reaction to pathologic stimuli. Its lack of specificity is apparent from the fact that the sedimentation rate is increased in greater or lesser degree in such varied conditions as pregnancy, neoplasms, and infections.

The sedimentation test derives its chief clinical value from the fact that it is a reliable and delicate indicator of activity, so that an increased sedimentation rate signifies an active process, whatever it may be, and a return toward the normal rate of sedimentation indicates a decreasing activity in the pathologic process. There are no specific sedimentation curves characteristic of any specific condition.

From the clinical standpoint the sedimentation curve has a very definite, though somewhat restricted, field of usefulness:

First, in gynecology as a means of determining when a tubal inflammatory process has become quiescent and thus most suitable for an elective operation. It has been suggested that there is a specific difference between the sedimentation rate in salpingitis and that in appendicitis, enabling a differential diagnosis between the two conditions, in that an increased rate appears earlier in salpingitis than in appendicitis. This observation awaits corroboration.

Second, in the management of ambulant tuberculosis where an increasing sedimentation rate is often the first indication of a reactivation of the tuberculous process.

Third, as a means of aiding in the differentiation of inflammatory from noninflammatory processes in, for example, the chest, in arthritides, and in the genitourinary system.

Fourth, as suggesting pregnancy in the absence of neoplasms and infectious processes.

Fifth, in distinguishing organic from functional disease in the gastrointestinal tract. It is a wise axiom never to diagnose a functional neurosis in the presence of an abnormal sedimentation rate.

Finally, there is evidence to suggest that, for reasons unknown, the sedimentation rate is either normal or decreased in pertussis, even though this is an acute febrile disease. Hence, in the presence of cough associated with a leucocytosis and marked lymphocytosis a coincident normal or decreased sedimentation rate suggests pertussis as a diagnostic possibility.

The sedimentation test has been applied in the presence of almost all known diseases, and in the early days of its use many statements were made which the test of time has failed to substantiate. While its mechanism is still largely unknown, it is practically certain that it is definitely related to tissue destruction, which, of course, explains its innumerable variations in innumerable conditions as a measure of activity.

It should be emphasized that inferential deductions drawn from the sedimentation rate are best and most safely and accurately based, not upon a single determination but upon the trend of a series of determinations.

AGGLUTINATION REACTIONS

Following the World War the rapid increase in the number of individuals vaccinated against infections of the typhoid group complicated somewhat the clinical interpretation of agglutination reactions. The problem appeared to become still further complicated by the more recent studies indicating that, in accordance with certain specific differences in typhoid strains, there were differences in the type of agglutinins produced and, consequently, in the character of the resulting agglutination reactions.

The discovery as long ago as 1917 that colonies of a pure culture of bacteria might vary in their appearance, some being smooth and shiny and others rough and more or less opaque, was soon followed by appreciation that these cultural differences were accompanied by certain other differences, among which were perceptible differences in the type of agglutinins produced by these two bacterial types.

When a suspension of a smooth or S-type organism is brought in contact with a specific agglutinating serum, the resulting agglutination takes the form of large flocculent clumps. When a suspension of a rough or R-type strain is agglutinated, the agglutination takes the form of small compact granules falling to the bottom of the tube.

S-agglutination occurs rapidly, settles slowly, and is flocculent in character. R-agglutination forms slowly, settles rapidly, and is granular in appearance.

Without entering into the many minutia of these studies it suffices to say that it has now been conclusively demonstrated that the granular type of agglutinin is thermostabile and of somatic origin, while the flocculent type of agglutinin is thermolabile and flagellar in origin. At first glance all this might seem to be of little or no clinical importance, but its practical significance becomes apparent from these facts:

1. Formolized suspensions for agglutination reactions respond mainly to the flagellar type of agglutinins, while alcohol-killed suspensions respond mainly to the somatic type of agglutinins.

2. While in the majority of instances both flagellate and somatic agglutinins are produced in typhoid fever, in some only the somatic type of agglutinin is produced, in which case there would be little if any response with a formolized agglutination suspension.

It thus appears that the thorough study of diseases of the typhoid group by means of the agglutination reaction may in the future necessitate the routine

use of both alcohol-killed and formalin-killed suspensions in order that both types of agglutination may be detected and quantitatively measured.

This becomes of clinical interest because of the fact that, in a general way, the agglutinins formed as a result of typhoid vaccination are largely of flagellar origin, while those resulting from an actual infection are largely of somatic origin.

Agglutination of the alcohol-killed suspension, therefore, usually suggests typhoid fever or infection with a closely allied species, while agglutination with a formalinized suspension, and hence of flagellar type, suggests one of three possibilities: the patient has typhoid fever, he has had the disease in the past, or he has been vaccinated.

The question is sometimes asked, "What strength of agglutination titer is diagnostic or suggestive of infection?"

The answer is clear and definite: No arbitrary titer can be selected which is positive in the diagnostic sense or below which it can be regarded as diagnostically negative. An agglutination reaction is simply one item of evidence to be considered in relation to all the other evidence available.

Certain general principles may be laid down, however, to govern the clinical utilization of this procedure:

1. Given a patient presenting symptoms suggestive of enteric fever—which, it must be remembered, includes infection with any of the organisms in the Salmonella group (of which there are about twelve species capable of causing continued fever)—a negative reaction to the suspensions used may signify that: (a) the patient does not have enteric fever; (b) that the specimen was taken before agglutinins appeared; (c) that the disease is of unusual type and caused by an organism not represented in the suspensions used. The first two alternatives are differentiated by a repetition of the test; the last by a resort to attempts to isolate the organisms by culture.

2. A negative reaction in the second or third week of the disease is of definite negative diagnostic value and repeated negative reactions render the clinical diagnosis unlikely.

3. A positive reaction must be considered in relation to (a) the distribution of agglutinins in the normal population, (b) the stage of the disease at which the test is made, and (c) the history as regards vaccination.

4. As is usually the case with many laboratory procedures, the trend of a series is of greater value and significance than a single determination.

5. Quantitative reactions are far more clinically valuable than tests with one or two arbitrarily selected serum dilutions.

6. It is a wise precaution to include a suspension of *B. abortus* in the routine Widal set-up and to use a *B. tularense* suspension in the study of any obscure continued fever.

BLOOD CHEMISTRY

Discussion of this phase of laboratory investigation must needs be brief and may be prefaced by the statement that the average hospital laboratory expends much time, labor, and material in the conduct of chemical determinations of little clinical value and which furnish little or no useful information.

It may also be emphasized that requests for "blood chemistry" or "routine blood chemistry" are meaningless. Every requisition for blood chemistry determinations should be specific and should have in view the acquisition of specific information relating to various phases of functional efficiency applicable to the corroboration or negation of a diagnostic possibility. This, indeed, applies to all laboratory requisitions.

The following generalizations are applicable:

1. As the urea nitrogen, in health and disease, is always approximately 50 per cent of the nonprotein nitrogen, it is unnecessary to order both. Determination of the urea nitrogen is preferable as simpler and more rapidly performed.

2. Creatinin need not be determined unless the blood urea is over 30 mg. per cent.

3. There is only one real indication for the determination of uric acid: gout or the suspicion of gout.

4. Blood calcium determinations are routinely indicated only in the presence of tetany; sometimes in the presence of spasmophilia associated with rickets, inasmuch as the blood calcium in rickets, as a rule, is normal; rarely in fractures when nonunion is thought to be associated with disturbances of calcium metabolism; and infrequently as a method of regulating parathyroid hormone therapy.

5. Phosphorus determinations are useful only in the study of tetany and rickets.

6. Chloride determinations find their greatest value in the study of the toxemias associated with disturbances of gastrointestinal motility. They are of little value or significance in nephritis.

7. Indications for determinations of carbon dioxide combining power are: (a) diabetic patients with acetone in the urine, (b) uremia with nitrogen retention and dyspnea, (c) toxic patients receiving alkali treatment, (d) tetany of all types, and (e) disturbances of gastrointestinal motility with toxemia.

Blood Culture.—It is not only wise but profitable to resort to blood cultures in (a) any disease known to be characterized by bacteremia; (b) in any condition where the clinical picture and symptomatology suggest bacteremia as a diagnostic possibility; (c) in any sustained febrile reaction the etiology of which is obscure; and (d) in certain acute infections, such as pneumonia or meningococcic meningitis for example, in which the demonstration of bacteremia is of pronounced prognostic significance.

The success or failure of bacteriologic examinations of the blood is determined by a variety of factors, some of which arise from the inherent characteristics of the disease in question, others dependent upon the particular method or methods employed; still others related to the characteristics of the bacteria in question; and, finally, those arising from the circumstances under which resort is had to this procedure.

In those diseases characterized by bacteremia, of which the typhoid group is the classic example, it is well recognized that the earlier in the disease the culture is taken the greater will be the incidence of positive results. The reason for this lies in the fact that in every bacteremia the entrance of the bacteria is

from some initial focus, that this entrance may be, and probably is, more or less intermittent, and that the bacteria are more or less constantly being removed from the blood stream by the varied mechanisms of defense.

In bacteremia the bacteria do not make continuous circuits of the blood stream reproducing as they go. On the contrary, not only do antibacterial substances arise and travel with them, but they are subject to mechanical removal by filtration, by lymph nodes, by deposition in various tissues and organs which may later function as foci of secondary invasions, and by phagocytosis.

As a result of these interacting forces it is probable that there is a rise and fall in the bacterial content of the blood dependent upon repeated influx from the primary as well as the secondary foci and influenced by repeated attempts of the defense mechanisms to cleanse the blood stream.

It is apparent, therefore, that the time at which the culture is taken is of definite importance and definitely influences the result. The most propitious time is hence as soon as possible after the clinical signs suggestive of bacterial invasion of the blood appear, namely, chill, rigor, and rise of temperature.

While the clinical pathologist usually inoculates a blood culture into a variety of media calculated to secure growth even when the organism in question is selective in its cultural requirements, it is obvious that knowledge of the presenting possibilities enables him to vary his methods accordingly and so enhance the chances of success. It is not only advisable, therefore, but of definite value that requests for blood cultures be accompanied by the tentative clinical diagnosis or some statement concerning the clinical features of the case.

Under certain conditions, such as in puerperal septicemia, for example, the invading organisms not infrequently, if not strictly anaerobic, grow best under conditions of partial tension. All blood cultures, therefore, should include anaerobic and partial tension methods.

Blood cultures should be so devised as to furnish information, not only of the presence of bacteria in the blood, but some measure as to the degree of bacterial invasion. In a word, they should be quantitative, and this can easily be done so that reports can be made in terms of the number of colonies per cubic centimeter of blood, thus furnishing the clinician with an estimate of the gravity of the condition of value from the prognostic as well as the therapeutic standpoint. Such a procedure in later cultures also serves as a measure of the response to treatment.

As a rule, after a preliminary report after twenty-four hours, blood cultures are not finally reported upon until they have been held for seven days. Sometimes, however, especially in subacute bacterial endocarditis, cultures must be held for three weeks or more before the final report can be made. In this condition it is hence imperative that the clinical pathologist be aware of this diagnostic possibility in order that he may not discard his cultures too soon.

The rapidity with which growth occurs in blood cultures is determined both by the ease with which the bacteria in question grow on artificial media, and also by the number present in the blood at the time the examination is made. Growth, even of organisms relatively easily propagated, may not be perceptible, therefore, for some little time.

The significance of negative blood cultures is governed by various factors among which may be noted:

1. The character of the infection. In subacute bacterial endocarditis, for example, where the blood stream invasion is not only intermittent but often of minor degree, several attempts may be necessary before growth is obtained.

2. The time at which the culture is taken. The further from the time of the invasion, the lower will be the incidence of positive cultures.

3. The duration of incubation as well as the media and methods used are of definite influence and emphasize again the value of informing the clinical pathologist of the possible diagnosis.

The demonstration in a blood culture of organisms not commonly encountered in the skin is positive evidence of a bacteremia. When staphylococci are found, however, and especially when they are present in small numbers or only in one or two media, the culture should be repeated in order to rule out skin contamination.

Staphylococci in large numbers in all media are usually evidence of staphylococcemia, although it is wise to check this finding by a repetition of the culture.

Flocculation Tests.—The present inauguration of a campaign against syphilis has focused attention anew upon the flocculation reactions which have been developed in recent years as methods for the serologic detection and study of syphilis. That they are reliable, delicate and useful adjuncts in the serologic study of syphilis is generally recognized. What does not seem to be equally as generally emphasized, however, is the fact that, as is true of any reaction which depends upon an interaction between the patient and his disease, these procedures have their limitations.

Much has been made of the fact that flocculation tests may be rapidly performed and especial emphasis has been laid upon their simplicity of technique. Indeed, so much has been said of these qualifications that, by inference or even direct statement, the impression has been spread that anyone from the janitor to the serologist may satisfactorily perform such tests, and they are being widely hailed as "office tests" that anyone can do. In fact, a prominent biologic supply house is now advertising a "new" flocculation test which can be completed in ten minutes, which—according to the advertisement—requires little, if any, skill, and which can be performed for an average cost of two cents! And another general supply mail order firm advertises the equipment for one of the older methods for $9.75.

It seems advisable and timely to recall some pertinent facts.

In the first place, there is evident danger that the public, as well as the physician, may be led to believe that the Wassermann test, or its equivalent, is all that is necessary to diagnose or rule out syphilis, from which it follows—if some recent trends in the literature and especially in the advertising "literature" directed to the physician are to be believed—that anyone on the sole basis of a ten-minute-office procedure can label a patient with the stigma of syphilis and condemn him to from two to five years of treatment; or, on the contrary, release a potential focus of infection on the same grounds.

It is not unprofitable, in this connection, to recall, from an address by Dr. James Gregory, Professor of Medicine in the University of Edinburgh from 1790 to 1821, the following:

"I do not know, nor can I conceive, any human contrivance that can more effectually and irresistibly oblige the physician to study carefully the case of his patient; to attend to every symptom and change of symptom; to exert himself to the utmost for his patient's relief, and at the same time be as careful as possible in the remedies he employs; than to find himself under the necessity of giving a minute account of everything he has done in a very public manner and before a number of competent judges."

Under such circumstances it is not unreasonable to suppose that there would be some embarrassment attached to a diagnosis of syphilis largely based upon a ten-minute-test and ten cents worth of reagents!

Now, while it cannot be disputed that serologic procedures are of paramount importance in the study of this disease, it must be remembered that careful histories, meticulous examination, and their careful correlation are equally important and equally essential. The old adage to "be quick to suspect syphilis but slow to diagnose it" is still in good standing.

In the second place, while it is true that the flocculation tests are simpler in technique than the complement fixation tests, behind these simple manipulations are the same basic principles underlying the complement fixation tests, principles none of which are simple, all of which are intricate and complicated, and some of which are yet incompletely understood.

It is important that workers in the field of serology understand them, as far as they can be understood, because of the fact that *all* laboratory procedures may go awry at times, and it takes an experienced worker with a thorough fundamental training both to appreciate when trouble begins and especially to find and eliminate the cause.

In the third place, the laboratory procedure itself is never as important as its interpretation, especially in the borderline case, in which neither the reaction obtained nor the clinical significance to be attributed to it are clear-cut.

It seems questionable, to say the least, whether perusal of the advertising "literature" accompanying the outfits now offered for sale for the performance of these "office tests" can justly be regarded as conveying sufficient information to make of the reader an efficient serologist or a competent syphilographer!

One may also be permitted to doubt whether or not rapidity and cheapness would be the paramount factor in determining the diagnosis of syphilis on one's own blood, even though hailed as sufficient for the patient.

It must be remembered that while the diagnosis of syphilis, with all that it implies, is very easily made, it is far from easily removed. The point need not be labored.

Even so rambling and discursive a discussion as this will serve to indicate, in some measure, the diversity of the present avenues of approach to the study

of disease. It may also serve to suggest something of the many factors upon which the clinical utilization and interpretation of laboratory findings depend.

It may, perhaps, again be emphasized that laboratory procedures, although commonly—and somewhat unfortunately—spoken of as "tests" are, of course, not tests for the presence or absence of disease, but specialized phases of the examination of the patient. While this need not be emphasized to the physician, it may with profit be called to the attention of the patient. For, thanks to the advertising methods of the lay owned and lay operated commercial laboratories and to the implications surrounding the drugstore laboratory, the public has become more or less "laboratory conscious."

By this it is not meant that the public has become trained to expect of the physician the proper utilization of all that modern medicine has to give to the study of disease, but rather that there has developed a more or less widespread tendency to regard the laboratory and its procedures as a sort of mystic divining rod for the detection of disease.

Every clinical pathologist encounters individuals who come to the laboratory for a "urine test" or a "blood test" to find out whether or not they are healthy, and has been obliged to expend valuable time in explaining, not always successfully, the futility of such procedures unless they are deemed indicated by the physician and subject to his interpretation. The laity, perhaps, are not altogether to blame in this matter, for medicine, to no small extent, is still somewhat shrouded in the mantle of mystery which it inherits from the past.

The necessity in these modern days for extensive use of laboratory methods has, by what Webster has called "the fearful concatenation of circumstance," resulted in a mushroom growth of "laboratories" not infrequently owned, conducted, and manned by lay personnel, trained solely in the purely manipulative features of laboratory procedures. Indeed, all too often the laboratories clamoring for the physicians' "business" are aggressive in proclaiming that "the test is the thing" and seek by an obtrusive display of glittering apparatus and the clamorous use of technical patter to "varnish nonsense with the charm of sound."

It is not altogether surprising that the average layman, to whom the clerk in the drugstore, the good fellow who handles his favorite brand of cigars, and the physician alike are known as "Doc," should regard the technician as a medical oracle.

The physician, of course, knows that technical skill and experience, no matter how extensive, do not make a clinical pathologist. On the contrary, the value of the clinical pathologist in the study of disease is entirely dependent upon and entirely in proportion to his clinical as well as laboratory ability to aid, when called upon, not only in the selection of suitable laboratory studies applicable to a particular problem but also, at times, to aid in their clinical interpretation and utilization.

For so complex may clinical problems become that one is reminded sometimes of the words of General Wolfe in one of his dispatches to Lord Pitt: "There is such a choice of difficulties that I find myself at a loss how to determine."

STUDIES ON ANAPHYLAXIS WITH INSULIN*

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INTRODUCTION

INVESTIGATION of the physiologic effects of insulin has so completely engaged the research interest of workers in this field that little attention has been directed to its immunologic properties. These properties have been brought into increasing prominence by the reports of unusual reactions such as are seen in insulin-resistant[1] cases or in the insulin sensitive individuals who present urticaria, edemas, asthma, or other untoward manifestations following its injection. Minor allergic responses, such as "dwarf" urticaria are met with not infrequently, but despite the wide use of insulin, grosser sensitization, or anaphylaxis, is still uncommon enough to occasion several accounts of this condition annually.[2-6] Indeed, those who follow any large group of allergic individuals are uniformly impressed by the paucity of diabetics in their clinic population.[7-9] This observation has been the source of various theories regarding the diabetic constitution, and one wonders whether the latter is antithetic to the allergic constitution, or whether, genetically, the simultaneous appearance of the two traits constitutes a "lethal factor." One would not have the temerity to defend completely this latter hypothesis, especially in view of the actual, albeit rare occurrence of the two conditions in the same individual. Speculation regarding the mechanism of insulin resistance has also stimulated interest in its immune processes. Is insulin fixed in situ in such cases and slowly inactivated, or does the union of antigen and antibody immediately inactivate it? Does its combination with the fluids or tissues of the host under particular but as yet unknown conditions reduce its hypoglycemogenic effect? We do not presume in this communication to answer all these questions, many of which are in the present state of our knowledge necessarily rhetorical. However, some inquiry into the immunologic properties of insulin seemed indicated, and we report the results of our investigation on this point. Data from work pursuing the subject further will be recounted in a later communication. The present series consisted of several groups of animals of which one representative study is set forth below.

MATERIALS AND METHODS

The guinea pigs were males, weighing between 250 and 300 gm. Injections with Luer syringes were made intracutaneously with some possible subcutaneous leakage. Normal horse serum was obtained from Eli Lilly Company, whose commercial insulin U 40 was also used

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in the experiment. A highly purified commercial preparation of insulin U 223, derived from beef pancreas, and containing 1.880 gm. nitrogen per cubic centimeter was supplied by the Research Division of E. R. Squibb and Sons. We are indebted to Dr. E. M. K. Geiling and particularly to Dr. H. Jensen, of the Department of Pharmacology, Johns Hopkins University Medical School, for a generous quantity of powdered crystalline insulin used in these studies. Frederick Stearns and Company furnished us with their "crystalline" insulin U 40 (nitrogen content 14 per cent dry crystals—zinc content 1 mg. per 500 units). The omission of any reference in the tables to differentiate either type is intentional for purposes of simplification. The results with the two crystalline products were identical in every respect.

EXPERIMENTAL

We were first interested in the possibility of sensitizing guinea pigs to insulin. The method employed by one of us[10] in pollen sensitization was first used. This consisted in the use of a mixture of equal parts of normal horse serum and, in this instance, insulin U 40. The mixture was allowed to stand at room temperature for twenty-four hours and then kept at icebox temperature for further use. At four-day intervals guinea pigs were injected subcutaneously with 3 doses of 0.4 c.c. of this horse serum-insulin mixture, and after a total elapsed time of twenty-eight to thirty days, were ready for the test of anaphylaxis. Other guinea pigs were similarly injected with 0.2 c.c. commercial insulin alone (same lot). Several animals were lost through hypoglycemic shock or intercurrent infections, and necessary modifications in dosage may be observed in the tables. It was soon apparent that any sensitization induced to insulin was in no consistent manner dependent upon the horse serum component of the "combined" antigen, and this phase of the investigation was not extended. However, the initial anaphylaxis was greater in this group and is commented upon later. Other guinea pigs were divided into groups dependent upon the type of insulin used for sensitization and for shock, and upon the method of sensitization. They were shocked at three- and six-week intervals after the last sensitizing dose by intravenous injection into the exposed jugular vein. The amounts used were 0.2 to 0.4 c.c. of insulin, containing from 4 to 19 units as indicated. Reactions were carefully observed and were classified as described in the legend (Table I). Typical anaphylactic responses were observed within five minutes after injection. These consisted, in increasing order of severity, of nervousness, rapid breathing, scratching of nose, dyspnea, cyanosis, wheezing, cough, loss of posture, jactitation, convulsions, and death. Any reactions occurring after forty-five minutes were discounted as being possibly hypoglycemic and were considered as insulin shock. This latter type of reaction was slower and more gradual in its appearance. The animal first became listless, weak, and apathetic, and, if not given glucose, eventually exhibited convulsive movements. To combat the latter, intraperitoneal injections of 50 per cent glucose in physiologic saline were employed.

It can be seen from Tables I and II that animals receiving insulin alone react with relatively slight degrees of anaphylaxis three weeks after sensitization. When the insulin was "combined" with horse serum fair grades of anaphylaxis were obtained at the three weeks shock (Table I). However, on the second shock three weeks after the first intravenous assay, those animals which had received insulin alone in initial sensitization showed anaphylactic reactions at least equal to and generally greater than those in the horse serum-insulin group had shown at the three-week shock (Tables I and II). No experiments were made to bring out differences in the latter group at a second three-week shock.

The possible combinations of factors in this experiment suggested that the variations in the type of insulin used, the time, the number, and the route of injections might be of importance. Accordingly further experiments designed

TABLE I

THE DEGREE OF ANAPHYLACTIC RESPONSE WITH COMMERCIAL INSULIN, AFTER SENSITIZATION WITH INSULIN ALONE AND WHEN "COMBINED" WITH HORSE SERUM

GROUP	ANIMAL NO.	FIRST INTRAVENOUS SHOCK AT THREE WEEKS		SECOND SHOCK INTRAVENOUSLY THREE WEEKS LATER	
		TYPE INSULIN AND UNITS	REACTION	TYPE INSULIN AND UNITS	REACTION
Horse serum plus insulin 0.4 c.c. subcut. 3 doses	a	Commercial insulin 8 units	3+	Horse serum 0.2 c.c.	
	b		1+		
	c		4+		
	d		1+		
	e		4+		
	f		4+		
	g		1+		
	h		—	Commercial insulin 8 units	—
	i		1+		4+
	j		0		4+
	k		4+		
Commercial insulin 0.2 c.c. subcut. 3 doses	l	Commercial insulin 8 units	4+		
	m		2+		
	n		0		
	o		—		4+
	p		—		3+
	q				4+

Anaphylactic Response

4+ = lethal, with jactitation, convulsions, etc.
3+ = dyspnea, convulsions, prostrations with survival
2+ = dyspnea, cyanosis, wheezing, cough, loss of posture
1+ = cough, sneeze, scratching of nose, cyanosis, slight dyspnea, micturition, defecation, nervousness

TABLE II

THE DEGREE OF ANAPHYLACTIC RESPONSE IN GUINEA PIGS "SENSITIZED" WITH CRYSTALLINE INSULIN

GROUP	ANIMAL NO.	FIRST INTRAVENOUS SHOCK AT THREE WEEKS		SECOND SHOCK INTRAVENOUSLY THREE WEEKS LATER		
		TYPE INSULIN AND UNITS	REACTION	TYPE INSULIN AND UNITS		REACTION
A1 u 2-4-6 Subcut.	50	Crystalline insulin 8 units	—	Crystalline insulin	10	4+
	55		—		10	—
	58		—		10	4+
	60		—		19	4+
	67				19	4+
	49	Commercial insulin 8 units	—		10	3+
	70		—		15	—
	66		—		19	2+
	56		—	Commercial insulin	19	0
	73				19	2+
A2 u 6-4-4 Subcut.	1	Crystalline insulin 4 units	0	Crystalline insulin 4 units		4+
	3		2+			—
	7		1+			4+
	8		4+			
B u 6-4-4 Intrav.	17	Crystalline insulin 4 units	1+	Crystalline insulin 4 units		—
	18		3+			—
	19		0			4+
	20		0			0
	21		1+			—
	22		0			0
	23		2+			4+
	24		2+			1+

to demonstrate possible differences were carried out and are included in the tables (groups B, Tables II and III, and Table IV). Both crystalline and

TABLE III

THE DEGREE OF ANAPHYLACTIC RESPONSE IN GUINEA PIGS SENSITIZED WITH COMMERCIAL INSULIN

GROUP	ANIMAL NO.	FIRST INTRAVENOUS SHOCK AT THREE WEEKS		SECOND SHOCK INTRAVENOUSLY THREE WEEKS LATER	
		TYPE INSULIN AND UNITS	REACTION	TYPE INSULIN AND UNITS	REACTION
A1	52		—		—
u 2-4-6	53	Commercial	—	Commercial	0
Subcut.	63	insulin	—	insulin	0
	65	8 units	—	19 units	1+
	74				—
	51		—		4+
	57		—		0
	59	Crystalline	—		0
	68	insulin			0
	61	8 units	—		0
	71			Crystalline	3+
	69	Commercial		insulin	4+
		8 units		19 units	
A2	26		0		2+
u 6-4-4	27		—	Commercial	4+
Subcut.	28	Commercial	0	insulin	—
	29	insulin	0	4 units	—
	30	4 units	2+		1+
	32		4+		
B	41		0		—
u 6-4-4	42		0		—
Intrav.	43	Commercial	1+	Commercial	
	44	insulin	2+	insulin	0
	45	4 units	0	4 units	1+
	46		0		2+
	47		0		1+
	48		1+		2+

commercial insulin were used in every series. The animals shown in Table IV were "shocked" three weeks after the last sensitizing dose by a subcutaneous injection, and three weeks later by the intravenous route. The subcutaneous

TABLE IV

THE DEGREE OF ANAPHYLACTIC RESPONSE IN ANIMALS "SHOCKED" BY SUBCUTANEOUS ROUTE AND THREE WEEKS LATER BY INTRAVENOUS ROUTE

GROUP	ANIMAL NO.	FIRST SHOCK SUBCUTANEOUSLY AT THREE WEEKS		SECOND SHOCK INTRAVENOUSLY THREE WEEKS LATER	
		TYPE INSULIN AND UNITS	REACTION	TYPE INSULIN AND UNITS	REACTION
	10	Crystalline	0	Crystalline	2+
	11	insulin	0	insulin	4+
	13	4 units	0	4 units	—
	16		—		4+
Crystalline	33		0		4+
insulin	34		0		0
u 6-4-4	35	Commercial	0	Commercial	—
Subcut.	37	insulin	0	insulin	3+
	38	4 units	0	4 units	0
	39		0		—
	40		0		0

"shocking" was insignificant and apparently did not alter the grade of response at the second shock three weeks thereafter by the usual intravenous method. This would indicate that time rather than a previous intravenous injection of insulin is the factor of importance in establishing anaphylactic sensitization in these guinea pigs. Both groups B (Tables II and III) were given all 3 preparatory doses by intravenous routes, as well as the shock dose. These animals showed moderate but definite anaphylaxis at the first three-week assay but not significantly different from those sensitized by subcutaneous injections (groups A2, Tables II and III). At six weeks there were likewise no notable differences, though the crystalline insulin groups showed slightly more marked reactions. Within a given group the animals varied somewhat in their individual responses, but these variations from the experimental result were neither greater nor more frequent than is seen in any anaphylactic study in guinea pigs. Certain of the differences in the height of the reaction which at first glance seem inconsistent, are readily accounted for by differences in the unitage of the shock dose employed. The demonstration of precipitins for insulin was inconclusive. No Dale preparations were studied in this experiment.* Observations on skin tests with insulin in sensitized animals will be reported at a later date.

COMMENT

The ability successfully to sensitize guinea pigs with insulin as demonstrated in this experiment is not surprising. Jensen and others[11-15] have demonstrated that insulin is a complete protein and is without organ or species specificity; accordingly, we might reasonably expect it to exhibit the immunologic properties of a protein. The slightly greater anaphylactogenic potency of crystalline insulin has been mentioned above. It is possible that this variation may be intimately related to the relative differences in purification. Sulfur in the insulin molecule has been shown to be labile. The possible internal rearrangements which might take place in the insulin molecule in the presence of "foreign" material, such as specific serum and tissue products of the host of origin, are purely conjectural. Such rearrangements, however, might conceivably alter or modify its action. Efforts to control the route of sensitization and the factor of time disclosed that the use of the intravenous route made no appreciable difference in the degree of shock at the first three-week assay. It is seen, however, that the combination of insulin with horse serum produces a fairly consistent and moderate grade of anaphylaxis after three weeks (Table I).

Of considerable interest is the relative absence of fatal hypoglycemic shock from the insulin dosage used. The possible explanations might be: (a) During the sensitization the animals were not fasting, and were returned to cages where abundant food awaited them. (b) The combination of insulin with guinea pig serum might alter the potency of the insulin so as to reduce its effect (this is discussed elsewhere).[16] (c) In some instances liberal quantities of intraperitoneal glucose were used either to combat or forestall hypoglycemic shock.

*Since the completion of this work a study of the antigenic properties of insulin has been reported by J. H. Lewis.[19] By employing the Schultz-Dale technique, utilizing strips of uterus taken from virgin guinea pigs previously injected subcutaneously with 1 mg. doses of beef and pork insulin, he demonstrated a definite anaphylactic response. He thus showed that sensitization rested not on the proteins of the animal of origin (of the insulin) but apparently upon the insulin molecule itself.

(d) In guinea pigs showing no hypoglycemia and not given glucose the question of the antihormone effect of Collip[17] and others arises. However, reasonable doubts have been cast on the existence of antihormones by the work of Sulman with the gonadotropic substance.[18] A similar end result which may be indistinguishable from the so-called "antihormone effect" may theoretically be due to specific antibodies to the endocrine substance which unite with the antigen and thereby impede its action. Such antibodies have not been conclusively demonstrated and the explanation of this phenomenon remains to be elucidated.

Of practical significance is the fact that sensitization to insulin has been produced in the guinea pig. In the human being this sensitizing property of insulin might be seen to be more in evidence on resumption of therapy after a period of insulin freedom, which permits the sensitization to manifest itself, whereas such sensitization had been previously obscured by the frequent therapeutic (and incidentally desensitizing) injections. However, follow-up data from cases known to have received insulin intermittently would have to be studied to affirm or deny the validity of such a theory. Whether the extended use of crystalline and protamine insulin will produce more frequent or less frequent sensitizations to insulin remains to be seen.

SUMMARY AND CONCLUSIONS

1. Guinea pigs injected parenterally with commercial and crystalline insulin reacted with anaphylaxis on subsequent intravenous injection.

2. Guinea pigs sensitized by subcutaneous or intravenous injections of insulin reacted with varying degrees of anaphylactic shock at intervals of three and six weeks following sensitization. The first shock at three weeks was generally less severe than the second at six weeks, using the same shock dose of insulin.

3. When the guinea pigs were sensitized with a combination of horse serum and insulin, fair degrees of anaphylaxis to insulin were obtained from a shocking dose three weeks after the last sensitizing injection.

4. The questions of insulin resistance and insulin hypersensitivity are briefly considered.

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THE RELATIONSHIP OF INSULIN HYPOGLYCEMIA TO THE METHOD OF ADMINISTRATION AND THE TYPE OF INSULIN*

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THE recent discovery of protamine insulin and the increased availability of crystalline insulin have given origin to many reports concerning variations in their hypoglycemogenic effects. In a study of certain of the immunologic features of insulin, incidental observations were made which were considered worthy of record. For example, it was noted that those guinea pigs injected subcutaneously developed typical hypoglycemic reactions in one and one-half hours to two hours, whereas those receiving insulin (commercial or crystalline) intravenously during the sensitization period did not exhibit this phenomenon. The discrepancy was not easy to explain since the animals were treated under identical conditions and with the same insulin dosage. This observation led us to investigate the possible mechanism of such differences. The likelihood of a prompt combination of the injected insulin with the circulating blood suggested that a not too remote parallel might be obtained by a mixture in vitro of serum and insulin prior to injection. A study of the blood sugar responses following the injection of such a mixture and of other standard preparations of insulin was made and constitutes the basis of the following report.

EXPERIMENTAL

Materials and Methods.—Seven rabbits, average weight 3 kg., were used for the assays, the great majority of the determinations being done, however, on 4 rabbits. Each rabbit was injected at different times with 10 unit doses of the various insulin preparations recorded below by subcutaneous and intravenous routes. An interval of at least forty-eight hours elapsed between injections in any one animal. Some of the rabbits were used for 14 separate studies, and 2 animals were used only twice. A minimum of five studies was made in the

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case of commercial insulin intravenously and the maximum of fourteen was completed with commercial insulin subcutaneously. The number of experiments with any one preparation was determined largely by the consistency with which the curves could be reproduced. Those preparations which gave rather variable results were subjected to more determinations in order to arrive at a significant average level, whereas consistently similar responses on every assay obviated the necessity of more numerous determinations. Blood was drawn from the ear vein by a micropipette at thirty-minute intervals for blood sugar determinations by the micromethod of Hagedorn and Jensen.[1] The animals were interchanged as to types of insulin employed. Each period of observation lasted two and one-half to three hours, unless interrupted by hypoglycemic reactions requiring supplementary glucose. Equal parts of insulin (10 units) and guinea pig serum were mixed in a sterile test tube and allowed to stand at room temperature for several hours until used. No precipitate was noted. This procedure was carried out with both the commercial and crystalline insulins. Guinea pig blood was obtained by cardiac puncture, and after separation of the clot, the serum was drawn off and stored in the refrigerator for use as required. Fresh serum was obtained at frequent intervals. The insulin-serum mixtures were prepared before each new series was run.

The insulin was obtained from several sources, each of which had previously standardized the preparation according to the method set forth in the New and Non-Official Remedies. These brands of insulin are in common use everywhere and for that reason were not standardized by us. Dr. H. Jensen, of the Johns Hopkins University School of Medicine, kindly supplied us with the powdered crystalline insulin used in the early studies. One milligram of this insulin represents the equivalent of 24 units, and the solution was prepared by dissolving the calculated amount in 0.001 hydrochloric acid, so that 0.1 c.c. of the solution contained one unit of insulin. We are indebted to E. R. Squibb and Sons, who, through Dr. John E. Anderson, gave us two purified preparations of commercial insulin derived from beef pancreas:

(1) unit content = 223 U per c.c.
nitrogen content = 0.0075 to 0.01 mg. per c.c.
(2) unit content = 40 U per c.c.
nitrogen content = 0.0076 mg. per c.c.

The Frederick Stearns Company generously supplied a quantity of crystalline insulin, 40 units per c.c., with a nitrogen content of 14 per cent (dry crystals) and assay approximately 24 to 25 units per milligram. The results are based upon 54 experiments comprising 269 separate blood sugar determinations and are expressed as the percentage drop in blood sugar using 100 per cent as the fasting level (see Table I). Studies on protamine insulin were not made.*

TABLE I

THE AVERAGE LEVEL OF BLOOD SUGAR AT VARIOUS INTERVALS IN THE EXPERIMENT EXPRESSED IN TERMS OF PERCENTAGE OF THE ORIGINAL BLOOD SUGAR

TIME (MINUTES)	COMMERCIAL INSULIN 10 U SUBCUT.	COMMERCIAL INSULIN 10 U INTRAV.	COMMERCIAL INSULIN 10 U PLUS SERUM SUBCUT.	CRYSTALLINE INSULIN 10 U SUBCUT.	CRYSTALLINE INSULIN 10 U INTRAV.	CRYSTALLINE INSULIN 10 U PLUS SERUM SUBCUT.
38	40.12	42.95	35.67	44.35	48.01	30.35
76	24.71	50.06	39.15	53.03	69.42	33.81
112	49.20	67.54	46.48	49.95	53.97	42.47
148	47.12	56.18	41.08	48.84	55.18	37.10
218	54.35	38.30			43.80	
Average per cent drop per experimental period	46.70	51.006	40.095	49.042	54.074	35.932

*Since the completion of this work, H. Blotner has reported a similar study on protamine insulin in which he found the hypoglycemogenic potency of this preparation increased by the addition of serum.

RESULTS

Objectively, commercial and crystalline insulin produced definite hypoglycemic effects within one to two hours. The crystalline preparation was in general more effective. Commercial insulin plus guinea pig serum likewise elicited unmistakable insulin reactions. Crystalline insulin plus guinea pig serum produced the least number of reactions. In decreasing order of hypoglycemogenic potency were:

(1) Crystalline insulin intravenously, (2) commercial insulin intravenously, (3) crystalline insulin subcutaneously, (4) commercial insulin subcutaneously, (5) commercial insulin plus serum subcutaneously, and (6) crystalline insulin plus serum subcutaneously. (Insulin plus serum intravenously was not studied in this experiment.)

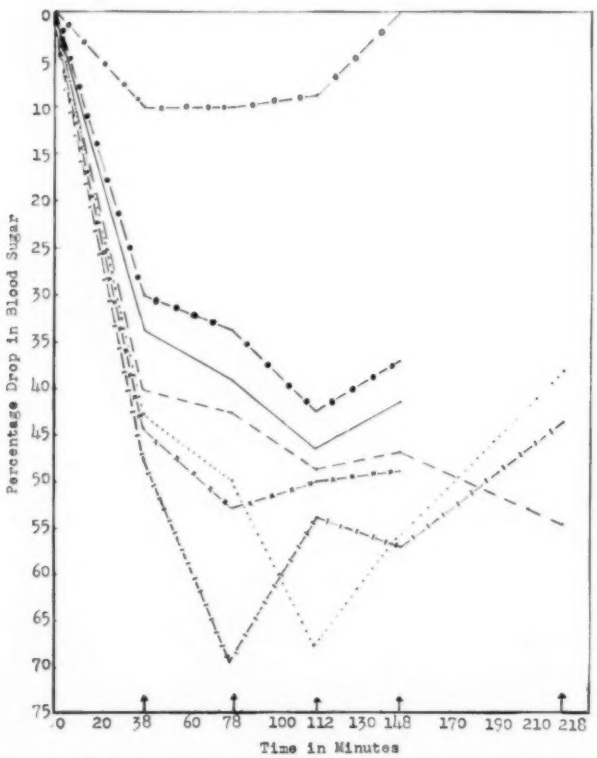

Chart 1.—Hypoglycemic effect (in the rabbit) of commercial and crystalline insulin alone and when "combined" with guinea pig serum, computed as a percentage drop in blood sugar.

—○—○—○— Guinea pig serum subcutaneously.
—●—●—●— Crystalline insulin plus serum subcutaneously.
———————— Commercial insulin plus serum subcutaneously.
— — — — — — Commercial insulin subcutaneously.
—×—×—×— Crystalline insulin subcutaneously.
. . . . . Commercial insulin intravenously.
—|—|—|—|— Crystalline insulin intravenously.

Several points of interest can be gathered from the chart of the blood sugar curves (Chart 1), obtained by averaging the findings in the various groups of studies. The most rapid drop occurs after intravenous injection of crystalline insulin which is maximal at an average of about seventy-eight minutes after injection. Commercial insulin intravenously acts only slightly

more slowly, reaching its maximum "low" at an average of one hundred and twelve minutes. This is in distinct contrast to the previous clinical reports (on subcutaneous injection[2-4]). The curves for subcutaneous crystalline and commercial insulin show that by subcutaneous route crystalline insulin is still, albeit slightly, more hypoglycemogenic than commercial. The "combined" insulin-serum preparations showed the least effect. Both were still distinctly hypoglycemogenic, the activity of crystalline insulin being slightly more reduced by such combination. The effect of the manipulative procedure without insulin was studied as a control, and no appreciable alterations in blood sugar were noted. Guinea pig serum used alone as an additional control caused a slight drop in the blood sugar level (see Chart 1) without observable reaction.

It was interesting to note the blood sugar level at which convulsions appeared (see Chart 2). The average level was about 35 mg. per cent, with the majority of convulsions appearing when the blood sugar ranged from 30 to 50 mg. per cent. Occasionally blood sugar levels within and below this range were not accompanied by convulsions. In two instances, reactions appeared

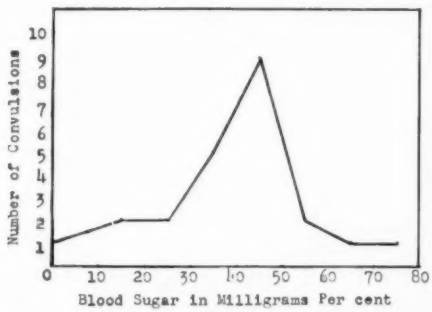

Chart 2.—The blood sugar level at which 24 observed insulin convulsions appeared in the rabbit.

when the blood sugar was 73 and 66 mg. per cent, respectively. Several observers[5, 6] have felt that the convulsive level in the usual rabbit assay is not uniform and that the appearance of convulsions does not necessarily coincide with a reduction of the blood sugar to 45 mg. per cent. It is not at all unlikely that of equal importance to the absolute level is the gradient, or rate of drop, of the blood sugar. The above results, however, agree fairly closely with the convulsive range of 50 to 26 mg. per cent obtained by Dotti.[7]

COMMENT

It is of considerable interest that, in contrast to the earlier reports in human beings in which crystalline insulin given subcutaneously was shown to have a slower but longer hypoglycemogenic effect, we find that in the rabbit the effect of crystalline insulin is not only in general more prompt but of less duration than the commercial preparation. This is also counter to our observations in guinea pigs that the intravenous injection of crystalline and commercial insulin was less likely to produce clinical hypoglycemia. In the rabbit the blood sugar curves show a greater drop with intravenous crystalline insulin than with any other preparation. Whether this may be explained on

the basis of a more gradual gradient in the drop in blood sugar, albeit to a lower level, or whether there is a true species difference between guinea pigs and rabbits in their reactions to insulin is not clear. An ever-present hazard in interpretation is the possible discrepancy in the unit assay by the manufacturers of the various types of insulin used. Since their methods are standard and their products in general clinical use, it was felt that re-assay would be unnecessary, particularly in view of the fact that these data were gathered secondarily to an investigation of the immunologic properties of insulin.

The reduced effect of insulin combined with serum also raises several questions. These have to do primarily with the mechanism of this reduction. Is there an "antigen-antibody" union which inactivates insulin? Does insulin combine with the constituents of the serum to reduce its hypoglycemogenic effect? Is there a so-called "antihormone"?[19] Or is insulin potency lost through enzymatic breakdown? It has been reported that normal human blood destroys the effect of crystalline insulin after incubation for several days at 37° C. Blood corpuscle-free serum has a similar though less intense effect. Inactivation of the blood serum by heating at 56° C. nullifies this destructive effect. Leucocyte-rich blood from leucemias has a more destructive action on insulin than normal blood. Similar studies have been made with blood from diabetic patients. Mauriac and Aubertin,[9] and Karelitz and his co-workers[10] have reported that diabetic plasma neutralizes insulin in vitro to a greater extent than does normal plasma. They felt that this neutralizing substance was trypsin. Black,[11] on the other hand, could not confirm these observations. Possibly trypsin forms inactive adsorption complexes with insulin; this undoubtedly occurs in vitro in strongly acid solutions.[12] Inactivation of insulin has also been accomplished by removing part of its sulfur content;[13] this is not too remote a possibility since Jensen and his associates[14] have shown that the hypoglycemogenic property of insulin is associated with certain di-thio (-S-S-) groupings present, in part at least, as combined cystine. This labile sulfur may unite in some way through molecular rearrangement with the serum with which it is mixed so as to alter appreciably the hypoglycemogenic effect. The existence of an anti-insulin[15, 16] has been hypothecated by several investigators, but as yet the existence of antihormones is without corroborated experimental foundation.[17] Experiments are now in progress by which some of the obscurity concerning this phenomenon may be clarified. With it are perhaps bound up some of the more bewildering phases of so-called insulin resistance. As a method of attack on the problem it is not without some promise.

SUMMARY AND CONCLUSIONS

1. The effects of commercial and crystalline preparations of insulin on the blood sugar of the rabbit were studied. Comparisons were made between the effects of subcutaneous and intravenous injection.

2. Crystalline insulin in the rabbit produced a greater drop in the blood sugar and one less sustained than did commercial insulin. This difference was greater on intravenous than on subcutaneous administration.

3. Guinea pig serum in combination with each type of insulin reduced the rate of hypoglycemogenesis but, unlike protamine insulin (current reports), did not substantially prolong it. Crystalline insulin suffered the greater reduction in potency.

4. The theoretical implications of the work are discussed.

The authors wish to acknowledge the valuable technical assistance of Miss Ann Prodehl.

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A COMPARISON OF THE ANTIPERNICIOUS ANEMIA POTENCY OF DEPEPSINIZED AND UNDEPEPSINIZED GASTRIC MUCOSA*

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IN ANOTHER communication[1] we are reporting some of our studies on pernicious anemia including an inquiry into the rôle of pepsin. We observed that depepsinized whole stomach mucosa, depepsinized fundus mucosa, and depepsinized pylorus mucosa, obtained from the hog's stomach under proper conditions for preserving antipernicious anemia potency and prepared according to the Greenspon process, were all practically inactive in the treatment of

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pernicious anemia when sources of extrinsic factor were excluded from the diet. When extrinsic factor was added there was minimal evidence of antipernicious activity in the patient fed with adequate amounts of depepsinized pyloric mucosa, but not in the patients fed with similar doses of depepsinized whole stomach or depepsinized fundus mucosa.

Since Greenspon[2] had claimed a higher activation of antipernicious anemia potency by depepsinization, we suggested[1] that the impairment of intestinal permeability may have accounted for our inability to confirm his conclusions. Moreover, we suggested that the process of depepsinization may not always result in the same end product and that whereas in one instance an active product may be obtained, in another the product may be inactive.

It occurred to us that the inactivity of our depepsinized products was so striking that another possible explanation seemed equally, and perhaps more, plausible than those just mentioned. In other words, though we permitted ourselves to question quite reasonably the degree of intestinal permeability, we could not doubt the adequacy of our sources of extrinsic factor. The possibility seemed apparent, therefore, that the process of depepsinization could have inactivated the intrinsic factor inasmuch as the response to products of known potency, though submaximal, was definite enough to rule out the factor of intestinal impermeability. We, therefore, decided to observe a patient with pernicious anemia treated with whole stomach material prepared as follows: The material was obtained from the slaughter house and preserved in dry ice until brought to the laboratory, when it was ground and immediately placed in a vacuum dryer, in which the temperature at no time exceeded 40° C. At the end of about thirty-six to forty-eight hours, it was further dried to remove the last traces of moisture over phosphorus pentoxide and then defatted with petroleum ether. In other words, the material differs essentially from the Greenspon process in that it is simply desiccated dried gastric mucosa without any attempt being made to remove the pepsin or to go through the various extractions as described by Greenspon.

If such a product were potent then we could assume that the process of depepsinization destroyed rather than enhanced the potency of these preparations. It is important to keep clearly in mind that even if the process of depepsinization inactivated the antipernicious anemia potency of these preparations that result does not necessarily mean that the absence of pepsin per se decreases the potency. In other words, a distinction must be made between the physicochemical changes occurring during depepsinization and the theoretic removal of pepsin without subjecting the mucosa to any changes which might influence the antipernicious anemia potency.

With these facts in mind we proceeded to treat a patient with typical pernicious anemia with undepepsinized desiccated whole stomach mucosa (see Table I).

The data in Table I (Patient J. L., aged 48 years) show conclusively that this preparation is completely satisfactory as a source of intrinsic factor. When compared to the responses of patients treated with depepsinized gastric mucosa (whole stomach, pylorus, fundus) as previously reported,[1] it would appear that Greenspon's process in some way results in loss

TABLE I

EFFECT OF ORAL ADMINISTRATION OF UNDEPEPSINIZED WHOLE STOMACH MUCOSA. DIET CONTAINS EXTRINSIC FACTOR. DESICCATED GASTRIC MUCOSA HAS ANTIPERNICIOUS ANEMIA POTENCY

DAYS OF TREATMENT	R.B.C. MILLIONS	HB. GM.	PER CENT	RETICULOCYTES PER CENT	TREATMENT DIET EXCLUDING INTRINSIC FACTOR AND INCLUDING EXTRINSIC FACTOR.*
1	2.45	7.9			"
2	2.60	8.9	60	2.5	"
3				2.2	"
4				3.0	"
5	2.70	8.6		1.9	"
6				1.5	" 24 gm. undepepsinized whole gastric mucosa q. d.
7				3.1	" " "
8	2.6	9.5		4.2	" " "
9				3.7	" " "
10				7.4	" " "
11	3.1	11.0		8.8	" " "
12				10.9	" " "
13				12.4	" " "
14	3.55	9.9		5.2	" " "
15				8.7	" " "
16	3.55	11.0		3.3	" " "
17				4.4	Liver extract 5 c.c. intramuscularly
18				2.4	" "
19					" "
20					" "
21	3.25	10.6			" "
22					
23	3.05	11.2		1.5	" "
24					
25					
26					
27	3.6	11.7		2.0	" "

*The extrinsic factor was provided in the form of meat and Brewer's yeast.

of activity of the gastric mucosa. However, we decided to repeat the administration of depepsinized preparations in increased dosage to two other patients in order to be sure that the maximum dosage of 40 gm. each day, as already reported, was sufficient. In the two following tables are recorded the data obtained when each of the two patients was fed with 50 gm. of depepsinized material each day. Both patients were ideal for the experiment not only because their counts were low but also because they represented typical uncomplicated cases (no neurologic signs or symptoms) of pernicious anemia. In view of the very low red blood count and hemoglobin readings we were advised to administer 30 gm. of our undepepsinized materials daily in order to be certain to obtain satisfactory responses. The equivalent dosage of ventriculin would be about 40 gm. daily. Our decision, therefore, to administer 50 gm. each day would insure adequate dosage.

Table II (Patient W. W., aged 63 years).—This patient's blood did not respond to adequate dosage of depepsinized pylorus mucosa administered orally. Oral and intramuscular therapy of known potency produced a characteristic response.

Table III (Patient S. M., aged 65 years).—Curiously enough, the depepsinized fundus material given in the dosage tabulated above was followed by a reticulocyte response of 7 per cent on the sixth day after the onset of treatment. Whether this response, in the light of our previous data,[1] was due to depepsinized fundus mucosa or to some extraneous factor or was spontaneous, it is difficult to be sure. The expected maximum reticulocyte percentage after desiccated stomach given orally is about 40 per cent. The response in the patient above is distinctly minimal.

TABLE II

EFFECT OF ORAL ADMINISTRATION OF DEPEPSINIZED PYLORUS MUCOSA. DIET CONTAINS EXTRINSIC FACTOR

DAYS OF TREATMENT	R.B.C. MILLIONS	HB. PER CENT	RETICULOCYTES PER CENT	TREATMENT
1	1.18	28	0.5	
2	1.15	28	0.5	Blood transfusion of 350 c.c. citrated blood*
3	1.50	34	0.0	House diet excluding liver, sweetbreads, and kidneys. 50 gm. depepsinized pylorus q. d.
4				"
5	1.25	32	0.0	"
6	1.37	35	1.3	"
7	1.20	30	1.0	"
8				"
9	1.80	36	0.4	"
10	1.61	29	0.4	"
11	1.75	32	0.2	"
12				"
13	1.50	30	0.3	"
14				Extralin tabs. 5 t. i. d. Lextron cap. 2 t. i. d. Liver extract 2 c.c. intramuscularly
15				" "
16	1.64	30	0.3	" "
17				" "
18				" "
19				" "
20				P. A. diet "
21	1.50	30	13.2	" "
22				" "
23	2.06	40	19.3	" "
24	2.20	40	7.9	" "
25				" "
26				" "
27	2.20	40 (6.4) gm.	4.5	" "
34	3.23	54 (8.6) gm.	0.4	" "
38	3.68	60 (9.6) gm.	1.1	" "

*The transfusion was given before it could be countermanded. However, it is apparent from the data that this procedure did not influence the response.

TABLE III

EFFECT OF ORAL ADMINISTRATION OF DEPEPSINIZED FUNDUS MUCOSA. DIET CONTAINS EXTRINSIC FACTOR

DAYS OF TREATMENT	R.B.C.	HB. PER CENT	RETICULOCYTE PER CENT	TREATMENT
1				25 gm. depep. fundus. Admitted late in evening
2	1,100,000	29	0.5	50 gm. depep. fundus
3				" " " "
4	820,000	31	3.5	" " " "
5				" " " "
6	1,020,000	30	7.0	" " " "
7				" " " "
8	980,000	26	5.0	" " " "
9				" " " "
10	1,020,000	28	4.0	25 gm. depep. fundus*
11	870,000	26	4.5	" " " " Transfusion given and intramuscular liver begun

*Patient reported severe nausea and inability to continue medication.

SUMMARY

The reticulocyte response to undepepsinized whole stomach administered orally conformed to the expected maximal as reported by Bethell and Goldhamer.[3] There was no reticulocyte response to depepsinized pylorus mucosa administered orally. In a previous report we found a minimal reticulocyte response but, on the other hand, there was no evidence in that report of antipernicious anemia activity in the fundus material. In the present instance (see Table III) there seemed to be some potency in the depepsinized fundus material administered. This response could, however, be due to the greater dosage of the substance administered or to the severity of the degree of the anemia or to the possibility, previously suggested, that the process of depepsinization may not always result in the same end product. However, since this patient had been active and remarkably free of distressing symptoms it seems likely that he must have been fortunate enough to have periodic, though very mild, spontaneous reticulocyte reactions, that is, minimal remissions.

Since we had already had occasion to treat two patients with pernicious anemia with depepsinized whole stomach without obtaining sufficient evidence of the presence of antipernicious anemia potency in it, we considered those cases a part of the present study in order to make the investigation complete.

The patients reported in this study were typical instances of pernicious anemia. There were no neurologic symptoms or signs. Achylia was present in each instance.

CONCLUSION

Cases of pernicious anemia treated with orally administered depepsinized gastric mucosa prepared by Greenspon's process show almost completely negative results. On the other hand, desiccated gastric mucosa, administered orally, produces the calculated maximum reticulocyte response. Therefore, it would appear that the process of depepsinization inactivates the antipernicious anemia activity of gastric mucosa. In other words, it apparently destroys the intrinsic factor since extrinsic factor had been provided in adequate amounts and the factor of intestinal permeability sufficiently accounted for. The exact rôle, however, of pepsin in pernicious anemia still remains a problem for further study inasmuch as our investigation has only proved that the *process* of depepsinization is responsible for the destruction of antipernicious anemia activity of these preparations.

NOTE: To Dr. John F. Anderson, Director, Biological Laboratories, E. R. Squibb & Sons, I owe my thanks for his cooperation in supplying me with the materials used in these studies. The expenses of this investigation were defrayed in part by a grant from the Julius Friedenwald Research Fund of the University of Maryland, School of Medicine.

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LABORATORY METHODS

THE IDE TEST FOR SYPHILIS*

A Comparative Study With the Kline Heated Serum Tests and Complement Fixation Methods, With Clinical Evaluation in More Than 1,000 Patients

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IN A RECENT publication[1] Ide described a new coloring method for the serologic diagnosis of syphilis. The present paper is a report on a comparative study of this test with the Kline heated serum tests and the complement fixation methods with clinical evaluation in more than one thousand cases. The blood specimens used in this study were collected from patients in a private dermatological practice and those attending skin clinics. An additional two hundred specimens were obtained from routine hospital patients.

The details of the test, as originally described by Ide, are as follows:

Materials.—1. Raw serum or whole finger blood.

2. Diluted Ide antigen. This is prepared by mixing 0.2 c.c. of Ide antigen with 0.6 c.c. of 2.5 per cent solution of sodium chloride in a small test tube. This mixture is shaken about thirty times.

3. Specially made hollow glass slides, having three depressions each, with a diameter of about 2 cm. and a depth of 2 mm., and a glass cover large enough to cover the entire slide.

4. Special dropping pipettes, so gauged that they deliver 0.03 c.c. of diluted Ide antigen, and others to deliver 0.03 c.c. of serum.

Technique.—1. Place 0.03 c.c. of raw blood serum in one cavity of the special hollow glass slide and spread out evenly over entire surface of depression.

2. Add one drop of diluted Ide antigen directly on to the serum.

3. Rotate the slide vigorously on a flat surface forward and backward as well as sideways for three or four minutes to insure adequate mixture. (In a later communication, Ide recommends prolonging the shaking time to eight minutes.)

4. Examine the results through a microscope with the low power. A positive reaction is evidenced by the appearance of purplish colored clumps, while in the negative reaction only tiny purplish bodies are seen.

Although Ide advocated the routine use of raw sera, he states that such sera obtained from syphilitic patients with varying amounts of treatment may occa-

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sionally give a negative reaction. In such instances he suggests heating the sera for twenty minutes. In a preliminary study, the Ide test was done with 26 raw sera. These sera were then inactivated at 56° C. for thirty minutes, and the Ide test repeated. It was found that the inactivated sera gave results which were as specific and considerably more sensitive (see Protocol 1). In this study, therefore, all the sera for the Ide tests were inactivated at 56° C. for thirty minutes.

PROTOCOL 1

COMPARISON BETWEEN 26 RAW AND HEATED SERUM IDE TESTS

RAW SERUM IDE	HEATED SERUM IDE
±	++++
++	+++
−	−
+++	++++
−	++++
−	−
−	++++
−	++
−	−
+	++++
+	+
−	±
++++	++++
−	−
−	+
−	−
−	+
±	++++
++++	++++
±	++
−	−
++++	++++
−	−
−	−
−	−
−	−

Furthermore, instead of employing the specially made hollow glass slides advocated by Ide, there were utilized the paraffin ring slides described by Green[2] for the Kline heated serum test.[3] These slides measure 2 by 3 inches, on which are mounted twelve paraffin rings, each with an inside diameter of 14 mm. This enables the serologist to do twelve tests on each slide. Since Ide advocated the preparation of 0.08 c.c. of diluted antigen, which is sufficient for thirty tests, and is satisfactory for use for thirty minutes, the serologist can do these 30 tests on three Kline test slides at one time. In addition, by using the Kline test slide instead of the hollow glass Ide slide, the tendency to evaporation is minimized.

Early in this study three hundred sera were tested on the Ide and Kline test slides with identical results. It was also found that with the Kline test slide as well as the Ide slide, the optimal rotation time was eight minutes.

The modified technique of the Ide test as used in this study is as follows:

1. Pipette 0.03 c.c. of inactivated serum into each paraffin ring.
2. Add one drop of diluted Ide antigen (approximately 0.03 c.c.).
3. Rotate the slides with moderate vigor for eight minutes.

4. Read degree of clumping through microscope (low power 16 mm. objective, eyepiece 12) at a magnification of about 120 times, with the light cut down as for the study of urinary sediments, and reported in terms of pluses according to the degree of clumping and the size of the clumps.

RESULTS

Comparison of 1,037 Ide and Kline Heated Serum Tests.—For this comparative study, blood specimens were obtained from 1,037 patients by venepuncture, and the sera heated at 56° C. for thirty minutes. The patients were divided into syphilitic and nonsyphilitic groups (see Table I). The former group consisted of 555 patients who either presented definite syphilitic lesions, or who admitted a previous syphilitic infection, or who were receiving antisyphilitic treatment at the time of this study.

TABLE I

COMPARISON OF IDE TEST AND KLINE DIAGNOSTIC TEST WITH 1037 SERA

SYPHILITIC GROUP (555 SERA)					NONSYPHILITIC GROUP (482 SERA)				
	AGREEMENT		DISAGREEMENT			AGREEMENT		DISAGREEMENT	
	ABSOLUTE	RELATIVE	POSITIVE KLINE DIAGNOSTIC NEGATIVE IDE	NEGATIVE KLINE DIAGNOSTIC POSITIVE IDE		ABSOLUTE	RELATIVE	POSITIVE KLINE DIAGNOSTIC NEGATIVE IDE	NEGATIVE KLINE DIAGNOSTIC POSITIVE IDE
Tests	436	103	14	2	Tests	444	37	0	1
Per cent	78.55	18.56	2.52	0.36	Per cent	92.11	7.67	0	0.21

The nonsyphilitic group consisted of 482 patients who presented no clinical or serologic evidence of syphilis. The sera of all the patients in this group gave negative results with both the Kline diagnostic and exclusion heated serum tests. The specificity and sensitivity of the Kline tests have already been established by the recent serologic conferences[4, 5] held in this country, under the auspices of the United States Public Health Service in cooperation with the American Society of Clinical Pathologists. The details of the Kline diagnostic and exclusion heated sera tests are given in Kline's book.[3]

The evaluation of the results of this comparative study was made according to the method of Kahn as follows: positive reactions, four-plus, three-plus, and two-plus; doubtful reactions, one-plus and plus-minus; agreement, positive or negative by both methods; relative agreement, positive or negative by one method and doubtful with the other; disagreement, positive by one method and negative with the other.

Of 482 nonsyphilitic persons whose sera gave negative reactions with the Kline diagnostic and exclusion test, 444 (92.11 per cent) gave negative reactions with the Ide test. In this group there were 37 (7.67 per cent) false doubtfuls and one (0.21 per cent) false positive reactions with the Ide test. This demonstrates that in this selected group the Ide test is somewhat less specific than the Kline heated serum tests.

Of the 555 syphilitic sera, there were 16 (2.88 per cent) disagreements, with a resulting total agreement of 97.11 per cent. Of the sixteen disagreements fourteen specimens were positive with the Kline diagnostic heated serum test and negative with the Ide test, while only two specimens gave negative reactions with the Kline diagnostic heated serum test and positive reactions with the Ide test. These two specimens, however, gave positive reactions with the Kline exclusion heated serum test. Thus if the Ide test alone had been used in this group, fourteen cases of syphilis would have been missed. This comparative study demonstrates that the Ide test is less sensitive than the Kline test.

TABLE II

COMPARISON OF IDE TEST AND COMPLEMENT FIXATION TESTS WITH 837 SERA

SYPHILITIC GROUP (347 SERA)					NONSYPHILITIC GROUP (490 SERA)				
	AGREEMENT		DISAGREEMENT			AGREEMENT		DISAGREEMENT	
	ABSOLUTE	RELATIVE	POSITIVE COMPLEMENT FIXATION NEGATIVE IDE	NEGATIVE COMPLEMENT FIXATION POSITIVE IDE		ABSOLUTE	RELATIVE	POSITIVE COMPLEMENT FIXATION NEGATIVE IDE	NEGATIVE COMPLEMENT FIXATION POSITIVE IDE
Source A					Source A				
Tests	98	33	11	2	Tests	200	22	0	1
Per cent	68.05	22.92	7.64	1.39	Per cent	89.69	9.86	0	0.45
Source B					Source B				
Tests	129	41	8	4	Tests	99	7	0	1
Per cent	70.88	22.53	4.39	2.2	Per cent	92.52	6.54	0	0.94
Source C					Source C				
Tests	11	8	2		Tests	149	10	0	1
Per cent	52.38	38.1	9.52		Per cent	93.13	6.25	0	0.62

Comparison of 837 Ide and Complement Fixation Methods.—The blood specimens for this group were obtained from three sources, and the technique of the complement fixation methods for each source is as follows:

Source A.—Total volume of serum used in test—0.55 c.c.

Test made with 0.04 and 0.02 c.c. serum with 2.5 units of guinea pig complement.

Antigen—0.2 per cent cholesterinized alcoholic extract of beef heart.

Amboceptor—diluted according to titration.

Corpuscles—sheep, diluted 1:20.

Sensitized cells consist of three parts of amboceptor and two parts of 5 per cent sheep cell suspension.

Incubation—First, overnight icebox, and second, fifteen-minute water-bath at 37° C.

Source B.—Total volume of serum used in test—0.5 c.c.

Test made with 0.05 c.c. and 0.02 c.c. serum, with 2 units of guinea pig complement.

Antigen—cholesterinized alcoholic extract of beef heart.

Amboceptor—Difco, diluted 1:1000.

Corpuscles—Sheep, diluted 1:20.

Incubation—First, one hour water-bath at 37° C., and second, fifteen minutes water-bath at 37° C.

Source C.—Test made with serum varying in amounts from 0.1 c.c. to 0.05 c.c.

Complement—guinea pig, diluted 1:10 and used in amounts varying from 0.2 c.c. to 0.3 c.c.

Antigen—two plain alcoholic extracts of beef heart. One cholesterinized alcoholic extract of beef heart.

Incubation—First, thirty minutes water-bath 37° C., then two and one-half hours icebox, second fifteen minutes water-bath 37° C.

Amboceptor—diluted according to titration and used in amounts varying from 0.05 to 0.1 c.c., depending on amount of natural amboceptor present in each patient's serum.

Corpuscles—sheep, diluted 1:20, used in 0.5 c.c. amounts.

The results (see Table II) show that the Ide test was somewhat less sensitive and less specific (with three nonspecific positive reactions) than the complement fixation methods.

COMMENT

The Ide test[1] is a simple test and easily performed. The antigen consists of an alcoholic extract (95 per cent alcohol) of beef heart muscle containing 0.2 per cent cholesterin. To 100 c.c. of this cholesterinized alcoholic extract is added 5 c.c. of a 5 per cent solution of gum benzoin, and 0.1 c.c. each of a 1 per cent alcoholic solution of crystal violet and azure II. In this study (comparison of Kline and Ide tests) the Ide test (antigen supplied by Dr. Ide) done with this crude alcoholic extract of beef heart, gave only one (0.21 per cent) false positive and 37 (7.67 per cent) false doubtful reactions with sera from 482 carefully selected presumably nonsyphilitic persons. The Ide tests performed with a commercially prepared antigen, however, gave results that were considerably less sensitive than those obtained with antigen supplied by Dr. Ide.

Ide antigen is a plain alcoholic extract antigen which contains a number of substances some of which are responsible for nonspecific reactions. Kline antigen is the acetone insoluble fraction only of alcoholic extract and contains no water, alcohol, or acetone soluble impurities.

CONCLUSIONS

1. A modified technique of the Ide test is described.

2. The modified Ide test has been found to be somewhat less sensitive and less specific than the Kline heated serum tests and complement fixation methods.

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A RAPID METHOD FOR STAINING BLOOD SMEARS

A Modification of the Original Wright Technique

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THIS method is particularly useful to the general practitioner. It may, however, be employed in larger hospital laboratories where time is a factor. The stain I have used is the original Wright's stain. It may or may not be fresh, cool, or stale.

After the smear has been made, place it in a convenient position over a receptacle of distilled water, or hold it in a simple thumb forceps. I, personally, fill a six-ounce drinking glass three quarters full of distilled water, and rest the slide on the edges of the glass.

(1) Cover the smear entirely with the Wright solution in stock. Leave it on for about ten seconds.

(2) Ignite the stain and let it flame for about ten to twenty seconds.

(3) With a suitable object, a pencil, a small rod, or any other such article, cause the flaming slide to fall into the distilled water, or drop it from the forceps into the container of distilled water.

(4) Remove, dry, and it is ready for use.

Only the nucleated cells will stain deeply. It is of interest that the nuclear material shows very much more detail than the ones stained according to the classic procedure. The red blood cells, however, are stained poorly by this method. No attempt to study them should be made at all.

In surgical cases, where a differential blood count is called for at once, this method is probably the most rapid, and yet as efficient at least as any we have at present.

A thin to medium streaked blood smear is the best to use for this method of rapid staining.

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A PRACTICAL STAIN FOR THE SPIROCHETES OF SYPHILIS AND VINCENT'S ANGINA

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THE following method of staining *Treponema pallidum* and *Borrelia vincenti* is simple, rapid, and reliable.

Diagnosis of syphilis by *quick* staining of serum exuding from a scraped lesion, or of tissue juice drawn by needle from a deep portion of a syphilitic area is of value because *all* organisms present can be observed. The method described stains the syphilis germs in these fluids *deeply;* in cases where spirochetes are few in number and dark-field tests fail to reveal them, smears stained by this method show the organisms plainly. It also yields equally good results when applied to aspirated testis juice of rabbits that have been artificially infected with *Treponema pallidum.*

Demonstration of *Borrelia vincenti* in ulcerative inflammation of mouth and pharynx is made easy by this method of staining. The spirochetes stain as deeply as other bacteria present.

The procedure is as follows:

1. Make a direct smear of the syphilis serum or tissue juice on a clean slide. In case of Vincent's angina, spread the ulcerative material in a small loopful of water on a clean slide.
2. Air dry. Fix with heat.
3. Cover with N/20 HCl for ten seconds.
4. Wash in running water for five seconds.
5. Cover with Gram's iodine solution for five to ten seconds.
6. Wash.
7. Cover with aniline-gentian violet for five to ten seconds.
8. Wash.
9. Cover with Gram's iodine solution for five to ten seconds.
10. Wash.
11. Cover with aniline-gentian violet for five to ten seconds.
12. Wash, blot, and examine.

The following suggestions are in order: (a) Gentian violet without aniline oil may be used. (b) A very deep color can be secured by applying the iodine and gentian violet solutions a third time. In staining *T. pallidum* this procedure is often of advantage. (c) The stain is not permanent, but this does not alter the diagnostic value of the method.

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A COMPARISON OF METHODS FOR THE PRESERVATION OF THE HEMOLYTIC ACTIVITY OF GUINEA PIG COMPLEMENT*

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THREE or more guinea pigs are usually bled to obtain a uniformly satisfactory complement. Often some of this fresh complement is left over, and unless special methods for preservation are used the serum rapidly deteriorates. The use of suitable methods of preservation eliminates the necessity of bleeding animals each time complement is needed and allows utilization of guinea pigs for serum at times when large numbers are available. Since there is a wide divergence of opinion regarding suitable methods for preservation of complement, it seemed desirable to study some more widely used methods and modifications these suggested.

Shirvindt and Liberman[1] advocated the addition to fresh complement of 10 per cent crystalline sodium chlorate with 4 per cent crystalline boric acid for storage at room temperature, and for storage at 5° C., the addition of 10 per cent crystalline sodium acetate with 4 per cent crystalline boric acid, reporting both preserved complement equally well. Sonnenschein[2] stated that complement was better preserved by using 10 per cent sodium acetate with 4 per cent boric acid than by 10 per cent sodium chloride with 4 per cent boric acid. Mirdamadi and Giese[3] preserved fresh guinea pig serum satisfactorily by adding an equal amount of 12 per cent sodium acetate containing 4 per cent boric acid. Austin[4] found that a 25 per cent salt solution would preserve complement for two to three weeks when stored in an ordinary test tube at icebox temperature.

Ginsburg and Kalinin[5] found full complement activity retained for three to four months at room temperature when 10 gm. of sodium chloride with 4 gm. of boric acid were added to 100 c.c. of guinea pig complement, using these in dry form rather than in solution as most other investigators had done. The same workers[6] reported the use of 5 per cent strontium chloride with 4 per cent boric acid was satisfactory. Ruffner[7] reported that 10 per cent sodium acetate or sodium chloride with 4 per cent boric acid was the best of many preservative substances studied since complement so treated kept four weeks with little change. Complement diluted to 40 per cent with a 12 per cent sodium acetate solution made up in physiologic saline retained its activity for three months at icebox temperature, and for several weeks at room temperature, according to Rhamy.[8] He pointed out that acetate was not "antibacterial," since growth took place when serum was contaminated, but that it preserved complement in some way by a physicochemic action which involved entering into a loose combination with the

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Based on a portion of a thesis submitted to the Graduate School of the University of Maryland by the senior author, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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complement. Ronchese[9] added 0.04 gm. of sodium fluoride per c.c. of complement, and found that its hemolytic activity remained constant during the first five days; it decreased "two-fold" during the next five days, and "three-fold" during the third five days.

Kolmer[10] investigated sixteen methods for the conservation of complement. He concluded that sodium chloride (0.17 gm. for each c.c. of complement) and storage at a low temperature yielded the best results. He recommended the method of Neill, who added 0.1 c.c. of a saturated sodium chloride solution to each c.c. of fresh complement when dilutions of 1:10 were used, and reported satisfactory preservation for two weeks when treated complement was kept at a low temperature. Kolmer[11] advocated the use of 25 per cent sodium chloride when a complement dilution of 1:30 was used. The National Institute of Health[12] reported complement preserved by the addition of 0.1 c.c. of saturated sodium chloride solution to each c.c. of serum maintained a satisfactory titer for six weeks. Complement preserved with 17 per cent sodium chloride with 0.25 per cent chinosol to maintain sterility kept the activity of the complement at its original level for twenty days according to Glover.[13]

Using hydrogen, nitrogen, carbon monoxide, and carbon dioxide to displace air, Valley and McAlpine[14] found only carbon dioxide valuable in preservation, complement titer remaining unchanged for several months. Valley[15] explained this preservative action was due to creation of conditions favoring reduction and preventing oxidation.

Ruediger[16, 17] used glycerol and freezing for preserving complement and reported Rhamy's method of preserving by sodium acetate a failure. Norton, Barfield, and Falk[18] found that at 37° C. complement completely deteriorated in about three days; whereas at 4° to 6° C. the original titer was maintained for forty-eight hours. When frozen and held at –10° C., it was usable for two weeks. Complement kept at –12° C. retained its hemolytic power for six weeks according to Browning and Mackie.[19] Bigger[20] determined the loss of activity of guinea pig serum at 50°, 40°, 30°, 20°, 9°, and 1.5° C. and found more rapid losses occurred as temperature increased.

Dean[21] failed to preserve complement titer in a dried form after precipitation with alcohol and ether. Flosdorf and Mudd[22] reviewed the development of drying as a method for the preservation of complement and described a procedure and apparatus for the preservation of sera, miscellaneous proteins, enzymes, viruses, and bacteria in "lyophile" form. Complement preserved by this method was best stored at 2° to 6° C., and storage for at least ten months did not result in a detectable drop of titer. Eagle, Strauss, and Steiner[23] found complement dehydrated from the frozen state, using the Flosdorf-Mudd apparatus, retained its full hemolytic activity throughout the experiment (eight months) stored at 2° to 6° C. Boerner and Lukens[24] used "lyophile" complement prepared from the pooled sera of 10 to 50 guinea pigs and obtained greater uniformity, little waste, and avoided the necessity of obtaining fresh complement during the summer. These same workers later[25] reported that "lyophile" complement stored at 8° to 10° C. retained its full hemolytic power for twelve months, some deterioration appearing after a year.

Greaves and Adair[26] recently described a procedure for the desiccation of antisera, complement, and bacterial cultures, which were frozen by rapid evaporation in a high vacuum in the presence of phosphorus pentoxide. Pooled complement preserved by this process over a four-month period maintained a titer comparable to fresh complement.

EXPERIMENTAL

Materials.—Complement was obtained from healthy male or nonbreeding female guinea pigs, all one year old or more. These were housed in an animal house and fed a complete commercial feed, which was removed eighteen to twenty-four hours before bleeding. The sera of from 6 to 15 animals were pooled to make up the sample. The sample of Lyo Complement* was reported to have been prepared from the pooled sera of fifty males.

In obtaining the blood for complement, guinea pigs were lightly etherized and 2 c.c. of heart blood withdrawn. This was placed in a sterile agglutination tube, slanted, allowed to stand at room temperature for one hour and then placed in a refrigerator (7° to 9° C.) for twenty-four hours before the titer was determined. Each sample was centrifuged to obtain clear serum.

Corpuscles were obtained from a ewe, blood from the jugular vein being aseptically aspirated into a 30 c.c. syringe and immediately expelled into a sterile flask containing glass beads, shaken for about ten minutes to defibrinate the blood, and refrigerated. The corpuscles were washed the same day, three times or more, packed by centrifuging for twenty minutes, made up to a 50 per cent suspension, refrigerated, and used within two days. The corpuscles were made up to a 2 per cent suspension in saline for use in the tests.

The same antisheep hemolysin was employed throughout the experiment. Frequent titrations showed that no deterioration had taken place. Saline[27] was prepared by dissolving 8.5 gm. C. P. sodium chloride and 0.1 gm. magnesium chloride in cold tap water, making up to one liter in volume, and autoclaving at fifteen pounds' pressure for thirty minutes.

Various samples of complement, in 3 c.c. portions, were stored in sterile, rubber-stoppered, glass vials with the exception of samples preserved with carbon dioxide, which were plugged with cotton. One cubic centimeter samples were used for freezing experiments. Sera receiving 0.04, 0.05, 0.1, 0.17, or 0.25 gm. of a chemical preservative per cubic centimeter were considered as containing 4, 5, 10, 17, or 25 per cent, respectively. Time consumed for removal from the refrigerator, sampling, and returning was approximately two minutes for refrigerated samples. All samples of fresh complement were stored at both room and refrigerator temperatures unless otherwise noted. Room temperatures ranged between 21° and 26° C. and electric refrigerator temperatures between 7° and 9° C., except when the freezing compartment was used, which was approximately –8° C.

Complement Titration.—Complement was titrated by determining the least amount necessary to bring about complete hemolysis of 0.5 c.c. of 2 per cent sheep

*Printed circular LR-44 accompanying packaged serum, Mulford Biological Laboratories, Sharp & Dohme, Philadelphia, Pa.

cells with two units of hemolysin. Complements were returned to isotonicity in diluting them 1:10, 1:20, or 1:30 according to the method of preservation. Unless otherwise noted, a 1:10 dilution was used, the amounts ranging from 0.06 c.c. to 0.30 c.c., with a 0.02 c.c. interval. When other dilutions were necessary 0.1 c.c. to 0.6 c.c. of 1:20 and 0.1 to 0.9 of 1:30 were used, with a 0.05-c.c. interval. The total volume was made up to 3 c.c. with saline. Saline, hemolysin, and complement controls were set up for each titration, and results were read after one hour's incubation in a water-bath at 37° C.

In determining the effect of various methods of preservation on hemolytic titer, 0.30 c.c. of a 1:10 dilution, or its equivalent, was considered as the titer beyond which complement would be valueless in complement fixation reactions. All titration figures given refer to the use of complement in dilutions of 1:10. Tables I, II, III, and IV, contain the results obtained with the various methods of preservation, including the time at which the end points were reached, or at which the samples were depleted or contaminated.

FRESH COMPLEMENT

Controls.—Three samples of complement, in duplicate, were stored without the addition of any preservative. Unpreserved complement held at room temperature had decreased in titer beyond 0.30 c.c. by the third or fifth day, when titers were determined. Less decrease occurred in the refrigerator, a titer beyond 0.30 c.c. not appearing until the tenth or twelfth day, although probably occurring sooner since there was an interval of one week between titrations (Table I).

TABLE I

HEMOLYTIC TITER OF UNPRESERVED COMPLEMENT

(Results Expressed in c.c. of a 1:10 Dilution)

TEST	FRESH	STORAGE				
		PLACE	DURATION—DAYS			
			3	5	10	12
I	0.04	Refrig.	–	0.18	–	0.30+
		Room	–	0.30+	–	–
II	0.06	Refrig.	0.18	–	0.30+	–
		Room	0.30+	–	–	–
III	0.08	Refrig.	0.14	–	0.30+	–
		Room	0.30+	–	–	–

Carbon Dioxide.—Three samples of complement, two in duplicate, were preserved by displacing oxygen in the containers with carbon dioxide. The sample was placed in a cylindrical museum jar, a vaselined rubber gasket applied, and the top, which had two openings, was clamped shut. Carbon dioxide was added through one opening and oxygen was forced out through the other, and after approximately three minutes, when practically all oxygen was displaced, the openings were closed. Two sets were maintained at room and refrigerator temperatures while the third was kept only in the refrigerator. The titers of both samples kept at room temperature had decreased more than half at the end of the first week. Samples held in the refrigerator were superior to those at room temperature since the first sample maintained its original titer for three

weeks, and when depleted at thirteen weeks had dropped only 0.06 c.c. in titer. The other refrigerated samples gave good titers for three weeks and four weeks (samples 3 and 2 respectively).

Sodium Acetate.—*A 12 per cent solution of sodium acetate* in 0.85 per cent sodium chloride was prepared and sterilized. Duplicate samples were prepared by mixing four parts of complement and six parts of the acetate solution, the mixture containing approximately 8 per cent sodium acetate. A 1:20 dilution was prepared for titration. Our results would indicate that at room temperature this method was not useful, as the titer dropped 0.12 c.c. at the end of one week. At refrigerator temperature the original titer was approximately maintained for two weeks; after four weeks there was a difference of only 0.04 c.c., and after nine weeks only 0.07 c.c. Accordingly this method at refrigerator temperatures preserved complement for nine weeks when the original sample was sufficiently active.

Two samples of *10 per cent sodium acetate with boric acid* were prepared in duplicate, one by adding 0.1 gm. of sodium acetate and 0.04 gm. of powdered boric acid to each cubic centimeter of serum; the other, by substituting 0.03 gm. of crystalline boric acid for 0.04 gm. of powdered boric acid. This was done because some precipitate was noticeable in the first sample and the crystalline boric acid went into solution much more readily. The first sample at room temperature held its original titer for one week, and during the next two weeks the titer dropped only 0.02 c.c. The second sample dropped 0.04 c.c. at the end of one week without further decrease through the fifth week. A reasonable titer was maintained at room temperature for three weeks. Storage at refrigerator temperature gave better results, both samples dropping only 0.06 c.c. during six weeks' storage.

Sodium Chloride.—*A saturated solution of C. P. sodium chloride* was sterilized and one sample in duplicate prepared by adding 0.1 c.c. to each c.c. of serum, the mixture containing approximately 3 per cent sodium chloride. At refrigerator temperature the complement held its original titer for two weeks and although the titer more than doubled by the sixth week due to originally high titer the complement was still relatively active. At room temperature, the original titer was maintained for one week, more than doubled during the second week, and by three weeks reached the selected limit.

Samples prepared with 10 per cent sodium chloride with boric acid were treated as with 10 per cent sodium acetate with boric acid. A considerable difference between samples at room temperature was noted; the first deteriorated rapidly, while the second varied only 0.02 c.c. from its original titer over a period of five weeks. Held at refrigerator temperature, the first sample kept its original titer for two weeks, but doubled it during the third week, and at the end of seven weeks a difference of 0.10 c.c. was noted. The second sample showed a difference of 0.02 c.c. for the first six weeks and 0.06 c.c. at the end of eleven weeks. The two samples had the same titers at seven weeks, but the actual loss of potency was greater in the first sample, emphasizing that only sera of high titers should be preserved and stored, an observation frequently made during this experiment.

TABLE II

HEMOLYTIC TITER OF COMPLEMENT PRESERVED BY VARIOUS METHODS

(Results Expressed in c.c. of a 1:10 Dilution)

TREATMENT	PER CENT	FRESH	STORAGE																	
			PLACE	DURATION—WEEKS																
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Carbon dioxide (1)		0.08	Refrig.	0.08	0.08	0.08	0.10	0.10	0.10	0.10	0.10	0.12	0.14	0.14	0.14	0.14	depleted			
			Room	0.18	0.18	0.18	0.18	0.18	0.18	0.30+										
Carbon dioxide (2)		0.08	Refrig.	0.14	0.14	0.14	0.16	0.18	0.18	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	depleted
			Room	0.26	0.26	0.30+														
Carbon dioxide (3)		0.10	Refrig.	0.14	0.14	0.16	0.20	0.20	0.20	0.20	0.20	0.22	0.24	0.24	0.24	0.24	0.30+			
Sodium acetate*	12	0.06	Refrig.	0.08	0.08	0.10	0.10	0.13	0.13	0.13	0.13	0.13	0.18	0.18	0.18	0.20	0.20	0.30+		
			Room	0.18	0.18	0.20	0.23	0.30+												
Sodium acetate	10	0.06	Refrig.	0.06	0.06	0.06	0.10	0.10	0.12	0.16	0.20	0.22	0.22	0.26	0.30+					
Boric acid	4		Room	0.06	0.08	0.08	0.18	0.18	0.30+											
Sodium acetate	10	0.10	Refrig.	0.12	0.12	0.12	0.12	0.14	0.16	0.16	0.16	0.16	0.16	0.16	0.22	0.30+				
Boric acid	3		Room	0.14	0.14	0.14	0.14	0.14	0.16	0.30+										
Sodium chloride	Sat.	0.06	Refrig.	0.06	0.06	0.10	0.12	0.12	0.14	0.26	0.26	0.30+								
			Room	0.06	0.14	0.30+														
Sodium chloride*	17	0.04	Refrig.	0.05	0.10	0.10	0.10	0.10	0.10	0.13	0.13	0.13	0.13	0.13	0.13	0.13	0.18	0.23	0.25	0.30+
			Room	0.05	0.10	0.15	0.20	0.23	0.30+											

*Titrations made in dilutions of 1:20. Results converted to 1:10.

†Titrations made in dilutions of 1:30. Results converted to 1:10.

‡Titer of 0.27 c.c. maintained through nineteenth week, titer of 0.30+ reached during twentieth week.

TABLE II—CONT'D

Sodium chloride†	25	0.06	Refrig.	0.07 0.07 0.10 0.12 0.14 0.14 0.15 0.19 0.19 0.19 0.19 0.19 0.20 0.20 0.22 0.25 0.27‡
			Room	0.10 0.10 0.15 0.20 0.30+
Sodium chloride	10	0.06	Refrig.	0.06 0.06 0.12 0.16 0.16 0.16 0.16 0.22 0.22 0.22 0.22 0.22 0.24 0.30+
Boric acid	4		Room	0.26 0.30+
Sodium chloride	10	0.10	Refrig.	0.12 0.12 0.12 0.12 0.12 0.12 0.16 0.16 0.16 0.16 0.16 0.20 contaminated
Boric acid	3		Room	0.12 0.12 0.12 0.12 0.12 0.16 0.30+
Sodium chlorate	10	0.06	Refrig.	0.30+
Boric acid	4		Room	0.30+
Sodium chlorate	10	0.10	Refrig.	0.30+
Boric acid	4		Room	0.30+
Sodium fluoride	4	0.08	Refrig.	0.08 0.08 0.10 0.12 0.14 0.14 0.18 0.18 0.26 0.30+
			Room	0.30+
Strontium chloride	5	0.08	Refrig.	0.30+
Boric acid	4		Room	0.30+
Strontium chloride	5	0.10	Refrig.	0.30+
Boric acid	4		Room	0.30+
Magnesium sulfate	5	0.10	Refrig.	0.12 0.20 0.24 0.30+
			Room	0.24 0.30+
Freezing		0.10	-8° C.	0.12 0.24 0.28 0.30+

One sample in duplicate containing *17 per cent sodium chloride* was prepared by dissolving 0.17 gm. of sodium chloride in each cubic centimeter of serum. A dilution of 1:20 was prepared for titration. This preserved complement had approximately its original titer both at room and refrigerator temperature for one week, and by the end of the second week the titers were still equal but 0.06 c.c. poorer. After that, the titer of the sample stored at room temperature was far inferior to that held at refrigerator temperature, which assured preserving action for at least six weeks.

One sample in duplicate containing *25 per cent sodium chloride* was prepared by dissolving 0.25 gm. of sodium chloride in each cubic centimeter of serum. A 1:30 dilution was prepared for titration. At room temperature the titer remained within 0.04 c.c. of the original for the first two weeks. At refrigerator temperature the titer remained approximately the same for two weeks, and was slightly more than double at the end of six weeks. Although doubled, the titer then was of such strength that it could be considered usable, because again this original sample was of high titer.

Sodium Salts (Miscellaneous).—Two samples of complement preserved with *10 per cent sodium chlorate with boric acid* were prepared in duplicate by adding 0.1 gm. of sodium chlorate and 0.04 gm. crystalline boric acid to each cubic centimeter of serum. No preservative action was shown at either room or refrigerator temperature.

One sample in duplicate containing *1 per cent sodium fluoride* was made up by mixing 0.04 gm. of sodium fluoride with each cubic centimeter of serum. This method did not preserve complement activity at room temperature, but when stored in the refrigerator, maintained the original titer for two weeks. At the end of six weeks the titer was nearly double but still usable.

Other Salts.—Two samples of *5 per cent strontium chloride with boric acid* were prepared in duplicate by adding 0.05 gm. of strontium chloride and 0.04 gm. crystalline boric acid to each cubic centimeter of serum. No preservative action on complement was shown at either room or refrigerator temperature.

One sample in duplicate containing *5 per cent magnesium sulfate* was prepared by adding 0.05 gm. magnesium sulfate to each cubic centimeter of serum. Room temperature storage proved of no value, while at refrigerator temperature the original titer was maintained for one week, was half at the end of the second week, and by the fourth week it was over 0.30 c.c.

Freezing.—Six 1 c.c. samples were placed in the freezing compartment of an electric refrigerator at a temperature of approximately –8° C. For each titration a sample was thawed and diluted 1:10. This complement was usable at the end of the first week, but by the end of two weeks the titer had more than doubled. All of the above results are shown in Table II.

DEHYDRATED COMPLEMENT

Dehydration.—Eight 1 c.c. samples were dehydrated for *three hours* by the freezing-dehydration method of Flosdorf and Mudd.[22] Four samples were held at room temperature and four in the refrigerator. This complement when restored had a titer of over 0.30 c.c. at the end of the first month stored at room

temperature, and a titer of 0.26 c.c. when stored in the refrigerator, reaching a titer of over 0.30 c.c. at the end of the second month.

One 3 c.c. sample in duplicate dehydrated for *twenty-two hours** and stored in the refrigerator had a titer when restored at the end of the first month equal to the original fresh serum, the duplicate likewise giving this titer when examined at the end of four months. The 5 c.c. sample of *Lyo Complement* (Sharp and Dohme) used, the original titer and age of which were unknown, gave a titer of 0.12 c.c. The above samples were restored by the addition of distilled water, and diluted 1:10 for titration.

Dehydration and Salting.—One 3 c.c. sample of complement in duplicate was dehydrated twenty-two hours, first adding 0.15 c.c. and 0.30 c.c. of a 17 per cent salt solution respectively, and stored in the refrigerator. When restored to isotonicity and diluted 1:10, the sample with 0.15 c.c. had a titer at the end of

TABLE III

HEMOLYTIC TITER OF DEHYDRATED COMPLEMENT

(Results Expressed in c.c. of a 1:10 Dilution)

TREATMENT	FRESH	STORAGE				
		PLACE	DURATION—MONTHS 1	2	3	4
Dehydrated 3 hours	0.06	Refrig.	0.26	0.30+		
		Room	0.30+			
Dehydrated 22 hours*						
Sample 1	0.10	Refrig.	0.10			
Sample 2	0.10	Refrig.				0.10
Dehydrated 22 hours†	0.10	Refrig.		0.10		
Dehydrated 22 hours†	0.10	Refrig.				0.10
Lyo Complement (restored)	0.12	Refrig.	Age and titer of original unknown			

*Unsalted.

†Salted with 0.15 c.c. and 0.30 c.c. of 17 per cent sodium chloride respectively before dehydration.

the second month equal to the original fresh serum, the duplicate containing 0.30 c.c., likewise giving this titer when examined at the end of four months. The results on all the above dehydrated complements are given in Table III.

Restored Dehydrated Complement.—A portion of Lyo Complement, restored to its original volume but undiluted, was stored in 1 c.c. amounts at –8° C. in the *freezing* compartment of the refrigerator. When thawed at the end of one week a 1:10 dilution gave a titer more than double that of the original, and another examined after three weeks was over 0.30 c.c.

Another portion of Lyo Complement was preserved with *10 per cent sodium chloride with boric acid* by adding 0.1 gm. sodium chloride and 0.03 gm. of crystalline boric acid to each cubic centimeter of restored serum and refrigerated. When diluted 1:10 at the end of the first week, the titer was 0.08 c.c. lower, and by the end of the third week 0.10 c.c. lower.

*Through the kindness of Dr. Harry Eagle of the Johns Hopkins Hospital, four samples of complement (two unsalted, one salted with 0.15 c.c. of 17 per cent sodium chloride solution, and one salted with 0.30 c.c. of 17 per cent sodium chloride solution) were dehydrated for twenty-two hours.

A sample of restored unsalted complement was preserved with *17 per cent sodium chloride* by adding 0.17 gm. of sodium chloride to each cubic centimeter and stored in the refrigerator. When diluted 1:20 at the end of two weeks, the titer had decreased 0.05 c.c.

Carbon dioxide was used to preserve the remainder of the restored twenty-two-hour dehydrated sample to which 0.15 c.c. of 17 per cent salt solution had been added following the procedure used with fresh complement. This was stored in the electric refrigerator and restored to isotonicity before preparing 1:10 dilutions. The titer decreased one-half at the end of the first week and remained the same for six weeks.

The above results on restored dehydrated complement are shown in Table IV.

TABLE IV

HEMOLYTIC TITER OF RESTORED DEHYDRATED COMPLEMENT, FUETHER PRESERVED BY VARIOUS METHODS

(Results Expressed in c.c. of a 1:10 Dilution)

SOURCE	TREATMENT	PER CENT	TITER WHEN RESTORED	STORAGE									
				PLACE	DURATION—WEEKS								
					1	2	3	4	5	6	7	8	9	10
Lyo Complement	Freezing		0.12	−8° C.	0.26	0.26	0.30+							
Lyo Complement	Sodium chloride Boric acid	10 3	0.12	Refrig.	0.20	0.22	0.22	0.24	0.24	0.24	0.24	0.24	0.24	0.30+
Dehydrated 22 hr. (salted)	Carbon dioxide		0.10	Refrig.	0.20	0.20	0.20	0.20	0.20	0.20	0.22	0.30+		
Dehydrated 22 hr. (unsalted)	Sodium chloride	17*	0.10	Refrig.	0.15	0.15	0.18	0.20	0.20	0.20	0.20	0.20	0.20	0.24†

*Seventeen per cent sodium chloride was titrated in a dilution of 1:20 and results converted to 1:10.

†Depleted.

DISCUSSION

Results with carbon dioxide do not agree entirely with those of Valley and McAlpine,[14] but it did preserve complement in a relatively active state for at least three weeks at refrigerator temperature. Sonnenschein[2] found sodium acetate preserved complement better than sodium chloride. Our results indicate that if kept in the refrigerator a reasonable titer was maintained for at least six weeks when preserved with either, one not being superior to the other in one instance, while in another slightly better results were obtained with sodium acetate. At room temperature three of four samples were usable for three weeks. Sodium chlorate was not satisfactory, differing from the results of Shirvindt and Liberman[1] who advocated 10 per cent sodium chlorate with 4 per cent boric acid, but our results did accord with theirs, indicating the adequacy of 10 per cent

sodium acetate with 4 per cent boric acid. Likewise we can agree with Ruffner[7] that 10 per cent sodium acetate or sodium chloride, with 4 per cent boric acid, is among the better methods for preserving complement. Our findings would not indicate that complement kept at room temperature maintained full activity for three to four months when preserved in this manner as reported by Ginsburg and Kalinin.[5] Their samples, however, were stored in darkness, while ours were in a glass cabinet exposed to ordinary light in the room.

Magnesium sulfate had only limited preserving power, and that only at refrigerator temperature. Contrary to Ginsburg and Kalinin,[6] strontium chloride failed to show any preserving action, our results agreeing with Ruffner.[7] Saturated sodium chloride gave results that are nearly in accord with those of the National Institute of Health,[12] and indicate that a reasonably strong complement can be preserved for a six-week period if the original titer is high. A satisfactory complement was preserved six weeks by treating with sodium fluoride and refrigerating, results materially better than those reported by Ronchese.[9]

When complement was frozen in the ice compartment of an electric refrigerator, its titer would indicate that it could not be considered usable more than a week. Our results do not agree with those of Browning and Mackie,[10] who used a storage temperature of −12° C. and found hemolytic power retained for six weeks. A 12 per cent sodium acetate solution preserved complement at a usable titer for nine weeks when stored in the refrigerator, not equal to that found by Rhamy[8] but indicating, contrary to Ruediger,[16] that the method is valuable.

When a 1:20 dilution is desired, our results showed that 17 per cent sodium chloride, as reported by Kolmer,[10] was a useful method, reasonable amount of preserving action existing for at least six weeks at refrigerator temperature. Although approximately half of the activity was lost by the sixth week when preserved with 25 per cent sodium chloride and stored in the refrigerator, the titer was such that it was usable, agreeing with Kolmer.[11]

The activity of complement was preserved little if any when frozen and then dehydrated for only three hours. When unsalted complement was dehydrated twenty-two hours, a titer equal to that of the original sample was maintained for four months at refrigerator temperature, the longest time that any dehydrated complement was stored. Our results on dehydrated complement agree with those of Flosdorf and Mudd,[22] Eagle, Strauss, and Steiner,[23] and Boerner and Lukens[25] and were superior to those secured with the other methods we used. Samples preserved by salting and dehydration, when restored at the end of two and four months, had a titer equal to that of fresh serum, indicating that salting and dehydration was superior to any chemical methods tested.

Whether dehydrated complement after restoration to its original volume could be preserved by some method effective with fresh complement was next determined. If so, such a method might prove valuable for the small laboratory where some complement remained after restoration and immediate needs. Freezing in the ice compartment of a refrigerator was of limited value, similar to results obtained with fresh complement. Ten per cent sodium chloride and

boric acid did not preserve restored complement as well as undehydrated serum, nor did carbon dioxide or 17 per cent sodium chloride, although the latter was slightly superior. The complement could be considered usable after preservation and storage at refrigerator temperature by any of these three methods for at least three weeks, although half the complement activity was lost in all cases.

CONCLUSIONS

Unpreserved complements stored at room temperature were not usable when first titrated after three days, refrigerated samples not dropping as rapidly. Storage in a refrigerator was also much superior to room temperature for all preserved complements.

Strontium chloride and sodium chlorate with boric acid did not prove of any value as a preservative for complement at either room or refrigerator temperatures. Magnesium sulfate had limited preserving power at refrigerator temperature.

At room temperature a satisfactory titer of complement was maintained with 10 per cent sodium acetate with boric acid for three weeks, and with one of two samples of 10 per cent sodium chloride with boric acid. A saturated sodium chloride solution preserved satisfactorily for one week and a 17 or 25 per cent solution preserved for two weeks. At room temperature neither 12 per cent sodium acetate solution nor 4 per cent sodium fluoride preserved satisfactorily.

At refrigerator temperature a satisfactory titer was maintained for not more than nine weeks preserved with a 12 per cent sodium acetate solution, and for six weeks with 10 per cent sodium acetate (or sodium chloride) with boric acid, a saturated sodium chloride solution, 17 and 25 per cent sodium choride, or 4 per cent sodium fluoride.

When carbon dioxide almost completely replaced the oxygen in a closed container in which complement was stored, rather high hemolytic titer was maintained for at least three weeks at refrigerator temperature, but storage at room temperature was unsatisfactory.

Freezing maintained fresh complement at a usable titer for only one week and failed entirely to preserve restored frozen-dehydrated complement.

Preservation by freezing and dehydration of salted and unsalted complement for twenty-two hours, followed by storage in the refrigerator, gave the best results, maintaining the original titer for four months, the duration of storage. Dehydration for only three hours of unsalted complement was not satisfactory.

For preserving left-over restored frozen-dehydrated complement 17 per cent sodium chloride was most desirable if a dilution of 1:20 was desired. Carbon dioxide or 10 per cent sodium chloride with boric acid was found satisfactory when a 1:10 dilution was preferred.

Most methods for the preservation of complement did not maintain the *original* titers upon storage for any considerable time. Accordingly, fresh complement for preservation should originally have a high titer.

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A UNIVERSAL LABORATORY SHAKER*

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THE appliance here described was designed to meet the needs of the average clinical laboratory for an efficient shaking unit, universal in scope.

Though there are several shaking machines on the market and each performs its particular function in a more or less satisfactory manner, it is quite apparent that none make any attempt to include features applicable to each and every shaking operation.

Increasing inroads on the workers' time by various precipitin reactions for syphilis, vaccines, and allergic antigens all call for some mechanical aid whereby this burden can be somewhat lightened.

With the end in view of making an appliance that would embrace all laboratory operations of this nature, we made an inventory of the various uses to which such an apparatus would possibly be put. This list was headed by the Kline slide precipitin test for syphilis. This most serviceable reaction is coming to the fore and its well deserved popularity impressed me at once with the fact that first of all the appliance must be arranged to handle these slides. The Kline test is performed in many laboratories as an eliminative reaction to safeguard recipients in cases where transfusions of blood are required. Usually these are emergencies. It takes four tedious minutes to turn these slides by hand. We found that the technician could make excellent use of this precious time while a properly adjusted machine agitated the slides, more effectively than this could be done by hand, as it gives accurate, methodical shaking, eliminating the uncertain personal equation.

Next in importance, we considered the Kahn test. Here a vastly different type of motion is required. While in the Kline test, we require a gentle, semi-rotary motion, here we require a vigorous to-and-fro shake with the machine opened wide. Along this same line of thought we included vaccines and allergic antigens. The former needs no comment. All too often we spend many minutes attempting to shake smooth a rough vaccine. In regard to the latter, some if not all laboratories are being called upon, or soon will be called upon, to prepare various extemporaneous allergic extracts from time to time. Most common of these is house dust. It is the opinion of this writer, based upon observation, that while it takes several days to extract a dust with intermittent agitation, the molecular friction obtained by shaking one hour at high speed in the apparatus described will suffice.

Perhaps most important of all is the proper shaking of blood-counting pipettes. Errors in the performance of this little task properly lead those com-

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mitted in most clinical laboratories. Few indeed are the technicians equipped with sufficient perseverance to spend the required three minutes agitating these little tubes by hand. As a consequence many blood counts vary outside of the permissible clinical range. While we freely admit that in the average case, an error of a thousand or so in a white count is possibly of no grave importance, on the other hand, to those like ourselves, who are engaged in some allergic diagnostic work, this much error would completely nullify the value of our leucopenic index.

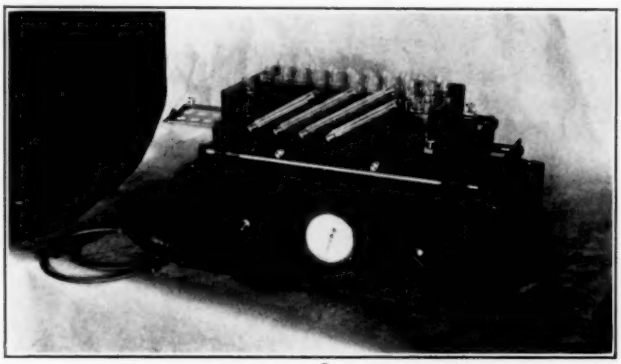

Fig. 1.

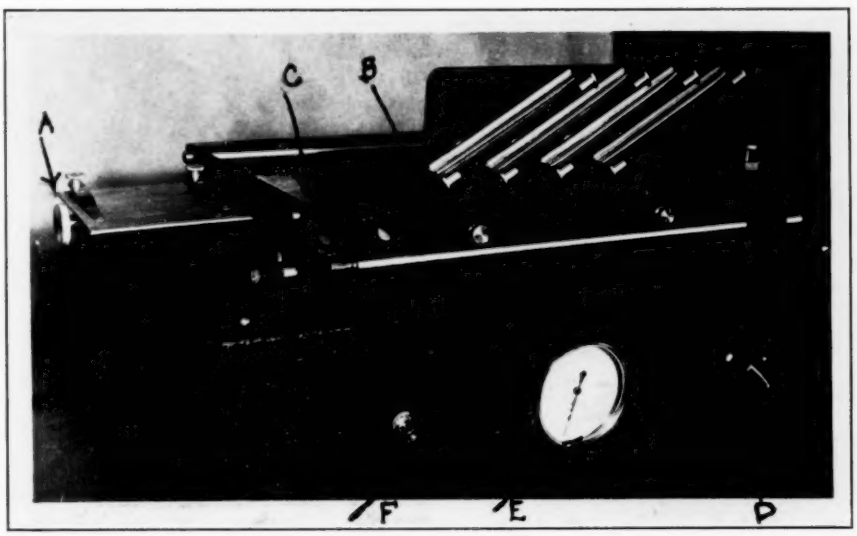

Fig. 2.—*A*, Kline slide holder and nut for its removal; *B*, rack for holding four blood count pipettes; *C*, main shaker table, for Kahn racks and miscellaneous articles; *D*, rheostat for speed control; *E*, speed indicator; *F*, snap switch, "off" and "on."

In this estimation, we pay some attention to a fall of 500 leucocytes and attach definite importance to the fall of 1,000.[1, 2]

It will at once be apparent to all workers that we required a vastly different stroke to properly shake these pipettes than we did to rotate a Kline slide or to rapidly agitate a rack of Kahns or a bottle of vaccine.

Summing up to this point, then, it will be seen that arrangements would have to be made to take care of at least four types of operation. The slow, gentle

rotation of the Kline slide a rapid, to-and-fro shake for Kahn racks, the adaptation of this same carriage that would hold these so that a small vaccine or other bottle could be accommodated. Finally, the problem of imparting to a rack of blood-count pipettes a combined shaking and rotating motion.

The apparatus finally assembled to perform these tasks is driven by a $\frac{1}{50}$ horse-power motor. Quite obviously there is an optimum speed for each shaking operation, consequently the motor is controlled by a rheostat and number of revolutions per minute noted on a speed indicator. This motive power and control are enclosed in a housing. Mounted on top of this is the shaking unit proper

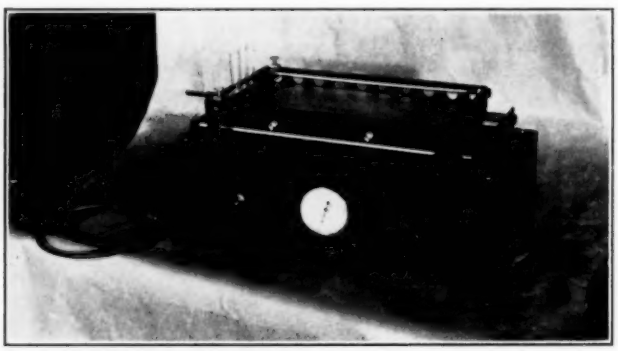

Fig. 3.

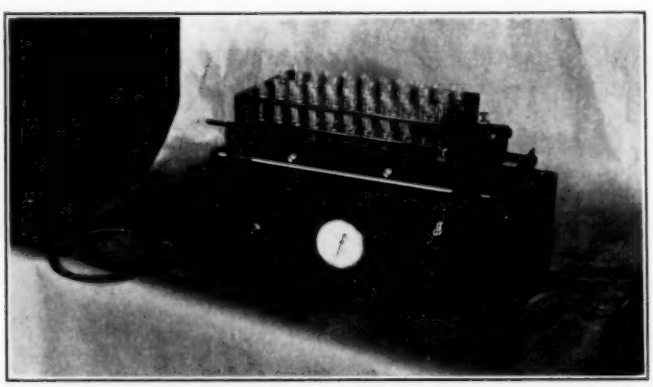

Fig. 4.

as illustrated. Fig. 1 shows the machine with all attachments in place. These can each be readily removed as required. In this picture one Kline slide is noted on left end. Arrangements can be made to place as high as four of these slide holders on housing. Fig. 2 shows close-up with key letters designating various parts. Fig. 3 shows all attachments removed except the sliding bar with which the shaking tray is equipped. This adjustable bar is designed to hold any object from two Kahn racks to one 2 c.c. test tube. It is freely movable and easily locked in place by two knurled nuts. Fig. 4 shows the appliance being used as a Kahn shaker, carrying two fully loaded racks.

The unit is exceedingly well balanced and vibration has been further reduced by mounting the apparatus on four vacuum cup rubber feet. Thus an annoying feature common to most rapidly moving machinery has been practically eliminated.

Optimum speeds have been determined by experiments to be as follows: for the rotation of Kline slides about 125 r.p.m.; for pipette shaking about 225 r.p.m.; and for shaking Kahns, vaccines, etc., 250 r.p.m.

CONCLUSION

A simple, practical, timesaving universal appliance, designed to meet every conceivable clinical laboratory need for an effective shaking apparatus has been discussed.

This apparatus is manufactured and distributed by Phipps and Bird, 916 E. Cary St., Richmond, Va.

Since completing this article we have had the opportunity to make several observations concerning the use of this appliance in connection with the Kline test. We are convinced that its use has added materially to the specificity of this reaction in our hands. This has been so satisfactory with the ordinary Kline diagnostic test that we have felt justified in eliminating the more complicated Exclusion test.

It has been noted that we have departed from the annoying doubtful reaction, since by the use of this mechanical rotation the reaction either breaks completely or else smooths out into a perfectly negative test.

We have found that gently mixing the serum and antigen, slightly spreading out within the ring, using a toothpick, has added materially to our results.

Optimum rotation time has been determined to be five minutes, and optimum speed at from 100 to 125 r.p.m.

Gratitude is hereby expressed to Dr. Warren T. Vaughan of Richmond, Va., Dr. Frank Wrenn, Medical Director of this Hospital, and to Mrs. Nan Stewart, my chief technician, for their aid and helpful suggestions in perfecting this instrument. I am also most grateful to the engineering staff of Phipps and Bird for their untiring zeal in assisting with the designing and assembling of the working model.

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A LOW-TEMPERATURE EVAPORATOR*

DANIEL S. STEVENS, PH.D., CHICAGO, ILL.

MANY substances, especially those of biological interest, are sensitive to temperature and so can best be isolated or concentrated with the aid of evaporation at low temperature. The machine shown in Fig. 1 has proved to be a rapid and convenient device for this purpose.

A metal tube (1) about 22 cm. in diameter and 150 cm. long is made into a vacuum tight chamber by soldering a cap (2) on one end and fitting a gasket-sealed door (3) on the other end. A plate (4) carries the dishes which hold the material to be evaporated. In our case these are five standard Pyrex trays 26 cm. by 16 cm. by 5 cm. Ten flat iron heating units are fastened to the under side of the plate (two in series under each dish).

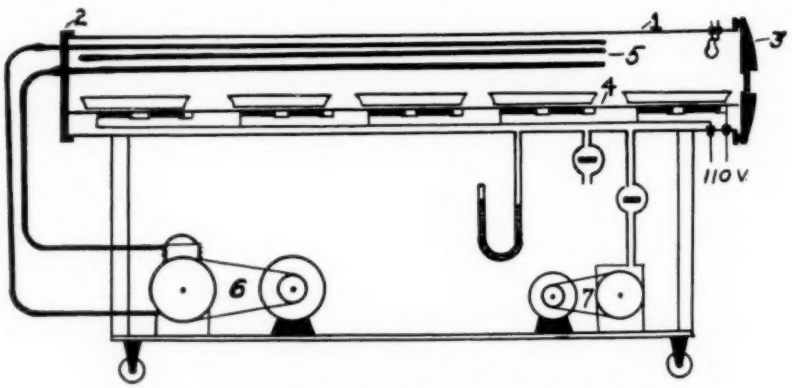

Fig. 1.—Low-temperature evaporator for laboratory use.

A refrigerating coil (5) occupies the top of the cylinder. This is made of three loops of 2 cm. copper tubing, having a length of 120 cm., which is 30 cm. shorter than the chamber, to leave space for tall flasks. The coil can be cooled to about –20° C. by the ¾ H.P. refrigerating unit (6). The chamber is evacuated by an oil pump (7).

To charge the machine, about 500 c.c. of solution is placed in each dish. The refrigeration unit, which is automatic in operation, and the vacuum pump are turned on. The interior is illuminated by an electric light, so that the dishes can be observed through a window in door (3). When the pressure in the chamber has been reduced to the vapor pressure of the solution, the liquid boils. The pump is then stopped and need not be operated again. The current through the heating elements is set at a value that will maintain the desired temperature in the dishes.

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The vapor from the solution condenses on the refrigeration coil. After the desired amount of evaporation has occurred, the dishes are removed and a long metal tray put in their place. The refrigeration unit is stopped and the coil defrosts into the tray.

If it is desired to evaporate the solution from the frozen state, the pumping is continued intermittently until it has turned to a solid. A thermoelement fastened to the side of a dish can work a relay in the heating circuit. A satisfactory element is a mercury thermometer with electrical contacts sealed into it. These can be obtained for operating at different temperatures, and standard relays have been designed for use with them. The thermoregulator is especially valuable when evaporation of the frozen material is required.

The machine described will evaporate in twenty-four hours about 2,500 c.c. of water at 8° C. About half this quantity of ice can be evaporated in the same time. The rate of evaporation rises rapidly with increased temperature and can also be increased by defrosting the coil more than once a day.

Vapors such as benzene and water will freeze on the coil without difficulty. Sometimes other volatile constituents are present. Ammonia gas will freeze with

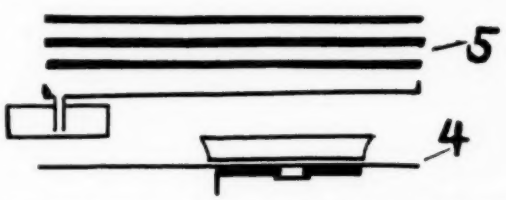

Fig. 2.—Arrangement for evaporating low-freezing liquids.

the ice without trouble. The vapor from an alcoholic solution may have a freezing point lower than the temperature of the refrigeration coil, especially since the vapor is much richer in alcohol than the original solution. In this case, liquid condenses on the coil and drips back into the dish.

However, alcoholic solutions can be handled by using the arrangement shown in Fig. 2. A tray with a reservoir is placed between the dish and the coil to catch the liquid that drips down. The presence of about 5 per cent alcohol in the solution is desirable for easy defrosting. This concentration can be frozen on the coil and the low melting layer next to the coil causes the ice to break away readily upon defrosting.

This machine is one of moderate size for laboratory use. A few machines have already been built of varying size. Their use on various products has shown that this device is not only convenient, but may be necessary to prevent undesirable changes in sensitive materials. The product obtained from the evaporation of ice is very finely divided. Solutions, either liquid or frozen, can be evaporated from the inside of ampoules.

The author is indebted to Dr. George F. Dick and Dr. Gladys H. Dick for suggesting the construction of this machine, and the Scarlet Fever Committee for its financial support.

COMBINATION MICROHEMOPIPETTE*

For Determination of the Sedimentation Rate, Packed Cell Volume, and Fragility of Erythrocytes, Especially Adapted for Use in Children and Small Laboratory Animals

Katsuji Kato, M.D., Ph.D., Chicago, Ill.

IN BOTH clinical and experimental studies of the blood such procedures as the estimation of the sedimentation rate, of the packed cell volume, and of the resistance of erythrocytes against various hemolytic substances, are increasingly being employed as a means of obtaining necessary information for accurate diagnosis and prognosis of anemic states. For the routine red cell count the use of standard blood diluting pipettes and counting chamber is now so well established that further improvement seems unnecessary. For the determination of the hemoglobin content of the blood several excellent methods are widely used with entire satisfaction, particularly those based on colorimetric principle of acid hematin (Newcomer, Haden-Hausser, Sahli, Hellige and Hellige-Wintrobe). But for the less frequently employed tests the instruments as well as the methods are so diverse and some even so cumbersome that great difficulty is experienced in selecting a procedure suitable for all purposes. A perusal of the rapidly growing literature dealing with the numerous devices and techniques for hematocrit, sedimentation, and fragility tests naturally suggests a need for the simplification and standardization of the instrument, incorporating the essential features inherent in each existing method. A combination or all-purpose microhemopipette here described has been devised in an attempt to fulfill this particular need. A serious attempt has been made to meet the four fundamental requirements which all improved laboratory methods should possess: (1) simplicity in both the instrument and in its manipulation, (2) maximum accuracy, (3) universal applicability, and (4) manifold usefulness.

In a comparatively brief period of time during which this new pipette has been used for various tests, I have found it to be satisfactory and timesaving, especially suitable in pediatric practice. The present report is merely a description of the instrument and the various methods of its use; the actual results of investigation, together with normal standards for each of the tests for which this pipette can be used, will be reported in subsequent publications.

DESCRIPTION OF THE PIPETTE

The pipette is a straight capillary tube of about a 5 mm. outside diameter, with a uniform bore of slightly less than 1 mm. diameter, and 12 cm. in length

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(Fig. 1*A*). The proximal end of the pipette tapers to a diameter of approximately 4 mm. so as to facilitate the attachment of the ordinary rubber suction tubing used for blood pipettes. The distal tip of the pipette is ground down to a diameter of approximately 2.5 mm. As measured from the distal tip, the portion actually used for observation is from 9 to 10 cm. in height, and is graduated into 100 equal parts of about 1 mm. each. The content of the bore so graduated is calibrated to hold exactly 50 c.mm. of blood. Since the accuracy of this volumetric calibration is given first consideration in the manufacture of the pipette, the actual height of the used portion of the bore varies inversely

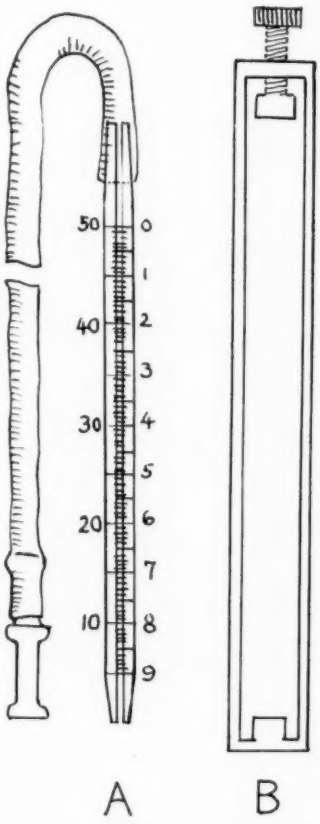

Fig. 1.—Combination microhemopipette (Kato). *A*, Pipette; *B*, metal frame. (Two-thirds actual size.)

according to the diameter of the bore in a given pipette. Thus by actual calculation it is found that only when the diameter of the bore is 0.79 mm. does the height of the used portion of the pipette approach 10 cm. for 50 c.mm. of volume content. Since absolute adherence to this particular ratio would be relatively costly in manufacturing the instrument and inasmuch as the small amount of deviation from this ideal proportion is not detrimental to the macroscopic interpretation of the results, a tolerance of 1 cm. in height is permissible without sacrificing the essential accuracy of the pipette. Two series of graduation numbers are etched on either side of the linear markings of the pipette, one series

on the left side (10, 20, 30, 40, and 50, reading upward) designating the volume content of the bore in cubic millimeters, and the other on the right side (0, 1, 2, 3, 4, 5, 6, 7, 8, and 9, reading downward) indicating the percentage figures when one cipher is added.

For centrifugation of the pipette a special metal frame has been evolved (Fig. 1*B*). This frame is constructed of a narrow metal sheet (brass or stainless steel), $\frac{1}{16}$ inch thick and $\frac{3}{8}$ inch wide, welded together at one end into a narrow rectangular shape, the total length being about 13 cm. and the width about 1 cm. The clearance space is thus just sufficient to accommodate the pipette. The inner surface of the bottom of the frame is provided with a small metal cup which receives the distal end of the pipette; the proximal end of the pipette is im-

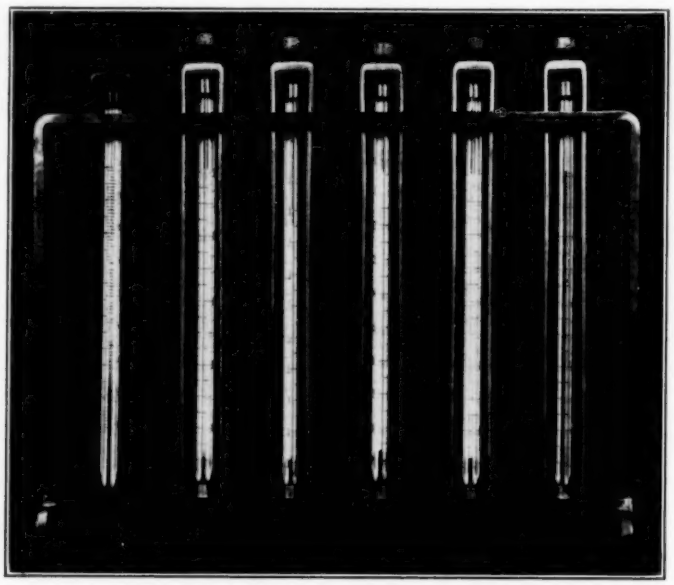

Fig. 2.—A set of six combination hemopipettes in metal frames placed in vertical position on a specially devised rack.

mobilized by a similar metal cup at the top of the frame, which is made adjustable by means of a small screw, approximately 2 cm. long, manipulated by a thumb piece outside the frame. Both the upper and lower metal cups are cushioned with small circular pieces of hard rubber to protect the tips of the pipette against breakage. The pipette is always inserted into the frame in a horizontal position, since the content of the pipette may be easily lost in vertical position. After the pipette is securely held in position, the frame and the pipette are set up in a suitable rack at vertical position (Fig. 2). For the sedimentation rate readings the fall of the erythrocyte column is noted at desired intervals; it is then placed in a 6 inch centrifuge cup and the packed cell volume obtained after fifteen to twenty minutes of centrifugation at 2,000 to 3,000 revolutions per minute. A special technique is required for the fragility test of erythrocytes, which will be reported later.

METHOD OF OBTAINING BLOOD SAMPLES

Capillary blood is recommended for performing various tests with the combination pipette, although venous blood, whenever available, can also be used with equal satisfaction. Venepuncture in infants and young children, or cardiac puncture in small experimental animals (rats, guinea pigs, and rabbits), is less convenient than pricking the finger or ear lobe. The object of the micropipette method is to enable the worker to perform various tests from the same small amount of blood readily obtainable by capillary puncture. From 0.2 to 0.3 c.c. of blood is sufficient for performing all necessary tests, including sedimentation rate, packed cell volume, and hemoglobin determination, as well as red and white cell counts. With this amount of blood even the fragility tests can be performed, providing every drop can be utilized without waste. Skill and speed in manipulation, acquired after a little practice, will enable the worker to perform a surprising number of tests by the use of this pipette, using as small an amount of blood as is obtainable from a capillary puncture.

As a receptacle for the blood sample a hanging drop slide with a central depression (15 mm. in diameter and 3 mm. in depth) is the most convenient. The surface of the depression is previously coated with a 2 per cent solution of oxalate mixture and dried. The oxalate mixture may be prepared by dissolving 0.8 gm. of potassium oxalate and 1.2 gm. of ammonium oxalate in 100 c.c. of distilled water (Heller and Paul[1]). The drops of blood from a puncture wound are received into the oxalate-coated cup of the slide and quickly agitated by means of a fine glass rod. The depression of a hanging drop slide of the above-mentioned dimensions holds, when filled to the brim, approximately 0.5 c.c. of blood. The worker must make sure that no coagulation has taken place before removing the sample to the laboratory for study. In a dry or warm season or climate the slide should be placed at once in a moist chamber (such as a Petri dish with a moist filter paper placed at the bottom) to avoid evaporation of the blood.

OTHER USES OF THE PIPETTE

Since the bore of the combination hemopipette is accurately calibrated, it can be used for obtaining precisely measured amounts of blood for various quantitative tests for which micromethods are available. The pipette is especially useful for removing samples of blood for use in the acid-hematin method hemoglobinometers. Thus for the Newcomer apparatus, exactly 10 c.mm. of blood is transferred into a test tube containing exactly 5 c.c. of 1 per cent hydrochloric acid. Similarly for the Sahli apparatus, exactly 20 c.mm. of blood is placed in a Sahli tube containing a measured amount of a N/10 hydrochloric acid. For other chemical analyses of the blood, particularly estimation of blood cholesterol, glucose, and minerals, the combination hemopipette offers a convenient means of obtaining accurately measured quantities of the blood.

In serodiagnosis of syphilis an increasing number of micromethods are being devised (micro-Kahn[2] and capillary Hinton[3] tests), and the technique of mixing the serum and the antigen in specified amounts will be greatly facilitated by the use of the combination pipette. More recently Isaacs[4] devised a cell count method

using bone marrow obtained at either autopsy or biopsy, and the technique of mixing the marrow material and the diluting fluids can be accomplished with relative ease by use of the combination pipette.

ADVANTAGES OF COMBINATION HEMOPIPETTE

The combination microhemopipette here briefly described is recommended to clinical and laboratory workers for more extensive trial and use on the basis of its distinctive advantages, summarized as follows:

(1) Being a capillary pipette, it requires only a small quantity of blood such as is obtainable by the routine skin-puncture method, without recourse to venepuncture in human beings or to cardiac puncture in small animals. This is an important practical consideration, particularly appreciated by pediatricians and by experimenters using small laboratory animals.

(2) The combination pipette with its metal frame, itself simple in construction, requires no additional laboratory equipment other than the ordinary type of centrifuge capable of a speed of 2,000 to 3,000 r.p.m. If for any reason the centrifuge at hand cannot develop a speed exceeding 2,000 r.p.m., a longer time must be allowed for complete packing of the red cells.

(3) The manifold usefulness of the combination pipette is attested by the fact that it can be utilized for the determination of the packed cell volume, of the suspension stability of erythrocytes, and of the fragility of red blood corpuscles against hemolytic substances. The pipette can also be used for removing precisely measured amounts of blood for various quantitative studies, particularly hemoglobin estimation by the acid-hematin method. It may also be used to advantage in mixing the serum and antigens in specified amounts in carrying out the microprecipitation tests in the diagnosis of syphilis.

(4) The universal applicability of the combination pipette to all types of hematologic examination in all age groups, but particularly in infants and small animals, facilitates performing the cell volume, sedimentation and fragility tests more frequently than is convenient with the more elaborate and cumbersome techniques, which in turn materially contributes to a more accurate diagnosis and prognosis of anemias. It is suggested that the combination microhemopipette be made a part of the regular hematologic equipment for routine procedures.

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USE OF DAPHNIA IN STUDY OF CATHARTIC ACTION*

WILLIAM TINSLEY, CHICAGO, ILL.

THE use of daphnia as an experimental animal is not new, as its availability, inexpensiveness, and ease in culturing make it an attractive form for study. Daphnids have been used widely in Russia[1] and some work has also been done in England with them[2] and in Canada,[3] mainly in the purely biologic field. Its reactions have been studied by these observers under the influence of various nutritive media, light, and heat.

Arno Viehoever[4] has, to our knowledge, been the first to use daphniae as a means of bio-assay. His published work on *Aloe Evaluation*[5] suggested the use of daphniae for the study and evaluation of catharties, and his method was tried at first; but we found it necessary to modify it to a certain degree to obtain more consistent results in our hands.

Daphnia magna, the preferred species for this work, is a minute crustacean belonging to the family Daphniidae, tribe 2, or Anomopoda (characterized by 5 or 6 pairs of trunk limbs, the first two pairs more or less prehensile) of suborder 1, the Calyptomera (carapace completely enclosing body and limbs), which belongs to the Cladocera, suborder 4 of the crustacean subclass Branchiopoda.[6]

CULTURING OF DAPHNIAE

After a study of the various types of culture media suggested by Banta[7] and Viehoever,[4] we have come to use the infusion prepared with dried sheep manure (Whizard brand), as suggested by Viehoever. It is to be understood at the outset that different batches of the culture medium will vary to a greater or less degree in constituents; but that, if it is prepared in the same way each time, any error is compensated for in the course of a sufficient number of observations.

Preparation of Culture Medium.—Whizard brand dried sheep manure is used in the proportion of 1 gm. to every 1,000 c.c. of tap water. A large container, preferably of earthen ware, is filled with chlorine-free tap water, and the required amount of the dried manure is placed in a small gauze bag and suspended in the water. After twenty-four hours the bag is removed. The water has usually turned a deep brown color at this time, but only a small number of infusoria and bacteria is present. The fluid is allowed to stand another twenty-four hours at room temperature, and at the end of this time a microscopic examination is made for infusorial forms, which not only serve as food for the daphnia, but also indicate that the medium is suitable for the development of minute animal forms. The growth should be quite abundant by this time. If it is absent and does not show up by the end of another day, the contents of the container are discarded and the container itself is thoroughly scrubbed, as the inhibition

*From the Laboratory of Pharmacology and Therapeutics, University of Illinois College of Medicine. Assisted by a grant from the Phenolphthalein Research, Inc.
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of infusorial growth after seventy-two hours is usually due to some contamination of the crock or water, sufficient to render the medium unsuitable for culturing of daphniae. We prepare and use for stock cultures fresh culture medium weekly, as the fauna and flora change on prolonged standing.

Placing the manure in a gauze bag eliminates the necessity of straining the liquid to remove excess organic matter that might cause decomposition of the medium. The resulting almost clear fluid is of light brown color and contains an abundant supply of fodder organisms necessary to maintain a healthy well-filled gut in the daphnia. When the daphniae are first placed in this medium, or when they are changed from one culture medium to the other, the gut may become emptied, and it may require a period of time to refill. For this reason daphniae which are once started in a sample of culture medium should be left in that sample, and water from *that* sample be used in the particular experiment with the daphniae taken from it. The effect on the metabolic rate of the daphniae resulting from changes in the culture medium due to aging of the medium and also due to changing to water of different sources or temperature, has been reported upon in an article by Warren.[8]

Because of the susceptibility of the daphniae to chlorine, it is necessary, as previously indicated, that the tap water used be free from chlorination.

It is advisable to check on the hydrogen ion concentration of the medium. Viehoever suggests the addition of an excess of calcium carbonate to the culture water to aid in maintaining an even pH.[4] In the course of experiments on the effect of pH changes on daphniae, we found that the culture medium itself has a tendency to maintain a pH of about 8.0. For more complete information on the effect of hydrogen ion concentration on daphniae, one might consult Klugh's work with buffer solutions of dibasic sodium phosphate and sodium acetate.[3]

Selection of Test Daphniae.—Mature daphniae are placed in large culture jars containing the medium.* Care must be exercised not to put an excessive number of daphniae in a given quantity of fluid. Crowding upsets the metabolic rate, probably due to insufficient oxygenation, resulting in lowered heart rate, increased susceptibility to unfavorable influences, shortened tail spine, and the production of males and of winter eggs. Crowding does cut down the number of young produced and daphniae from overcrowded cultures are unsatisfactory for assay work. It has been suggested that 40 c.c. of medium be allowed for each daphnia. We have found that a smaller quantity, but not below 10 c.c. per animal, can be used. Aeration is of advantage. This is done as follows: the jars containing the mature females are placed in a diffuse light (north window) away from fluctuations of the temperature above or below normal room temperature (68° F.). A capillary pipette is placed in each jar and connected with an air line. This is so regulated that a small bubble escapes every one or two seconds, and this is continued for hours at a time. These bubbles in traveling to the surface maintain a slow circulation in the medium and keep it sufficiently supplied with oxygen. The evaporated water should be replenished with distilled water from day to day. If the latter is added in small quantities, it has no deleterious effect on the daphnia.

*The culture jars we have found satisfactory measure 6 inches in diameter by 9 inches in height, containing about 4 liters.

Breeding of Test Specimens.—A mature gravid female (Fig. 1; recognized by the eggs within it) is placed in a liberal supply of culture medium, and multiplication is permitted to take place until a large stock of offspring has developed, which usually requires about two months. The gravid females are then pipetted into other jars, and these brood jars are inspected daily for newly born daphniae. As the daphniae retain the young in the brood sac until they are liberated as free swimming forms, it is a simple matter to pipette out the crop of young each day. The young daphnia is visible to the naked eye when born. The day's crop of young may be counted as they are removed and placed into other jars; these jars are then marked with the birth date and number of young and set aside. Such fleas as are not used experimentally are added to the stock

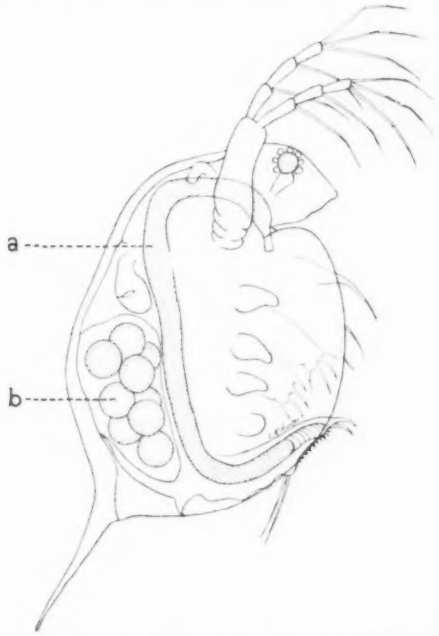

Fig. 1.—Mature gravid female daphnia. *a*, Gut; *b*, Ovum.

jars of gravid females to supply future cultures. The culture water in these brood jars is changed every other week.

All this preparatory procedure is important because only daphniae of a certain age are suitable for quantitative results.

McArthur and Baillie[9] have determined the heart rate to be highest and consequently the metabolic rate to be greatest on the seventh day. In experiments on the effect of age on consistency of results, we have found that this age gives the most consistent data, and that the results become more scattered with increasing maturity. As the daphniae become older the carapace becomes thicker and less transparent, rendering observation less easy. With maturity and the appearance of eggs in the brood sac, the results are still less reliable, due probably to changes in metabolism attendant upon the appearance of the eggs. Though seven-day daphniae would probably be the best for the purpose, we find their small size rather inconvenient. Daphniae ten days of age are large enough

to render observation easy; and, as the results with fleas of this age are still consistent, we agree with Viehoever[4] that this is the proper age at which to start observations.

TECHNIQUE OF THE TEST

Preliminary Qualitative Study.—It must, of course, be presupposed that qualitative observations, the method for which is discussed in another paper,[19] have previously been made; otherwise, a good deal of time may be wasted before one discovers possible fallacies of the test, namely: evacuation of the tract due to paralysis of the feeding mechanism without impairment of gut peristalsis. This can be discovered by finding that no food enters the upper part of the gut. It is essential that feeding continue throughout the observation period.

Preparation of the Test Solutions.—Ten-day daphniae with solidly filled tracts should be selected. About 50 c.c. of the same medium in which the specimens to be tested have been grown is decanted into a small beaker. The chemical to be tested is added to this decanted portion. In the case of many of the sparingly soluble materials with which we worked, we experienced difficulty in wetting the substance sufficiently to allow ready mixing with the water. In such

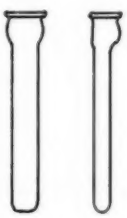

Fig. 2.—Daphnia observation tubes. (One-half actual size.)

cases the mixing is facilitated by agitating the dry substance with distilled water in a test tube until the material readily settles to the bottom on standing. The tube is then again agitated, and a quantity of the suspended material is added to the culture water. The latter is stirred up at intervals and then allowed to stand for about an hour to permit the excess material to settle. If an appreciable amount of the matter remains in suspension, it has a tendency to clump on the antennae of the daphniae, hindering their swimming movements and disturbing their normal feeding processes. At the end of one hour the culture water should be quite clear, the supernatant medium containing but few particles of the material in suspension, which is probably essential to maintain saturation.

Filtration is not permissible, because of the necessity of maintaining a sufficient number of fodder organisms to prevent "starvation evacuation," which might be misinterpreted as an emptying of the gut.

Filling of Observation Tubes.—The observation tubes (Fig. 2), devised by Viehoever, are thoroughly cleansed and numbered. We have found it most convenient to work with 10 to 20 tubes at each observation. A larger number excessively increases the period of time of actual inspection and may give rise to overlapping class intervals in the calculation of the final results. It is more desirable to run five daphniae in each of several solutions simultaneously than

to take observations on twenty in one solution on successive days, as sampling differences are then less likely to give erroneous indications on the comparative action of various substances.

The tubes are then filled with the supernatant liquid prepared as described above. A capillary pipette is the most convenient means of filling them. The tube is filled only to the point of flare at the top, leaving a small portion unfilled. The remaining test solution is decanted into a second beaker. The daphniae are pipetted into a third beaker with the smallest possible quantity of culture water. The beaker containing the decanted test solution is then emptied into the third beaker containing the daphnia and the time marked by starting a stop watch. This method insures that each daphnia is brought into contact with the test solution at the same time. A daphnid is then picked up in a glass tube (to which the bulb of a medicine dropper has been attached), and the water carefully dropped out until there remains only a drop or two containing the daphnia. One of the tubes is picked up and its lip placed against the edge of the dropper. The remaining water in the dropper is squeezed out and the daphnid carried into the observation tube. The transfer of the daphniae can be accomplished in

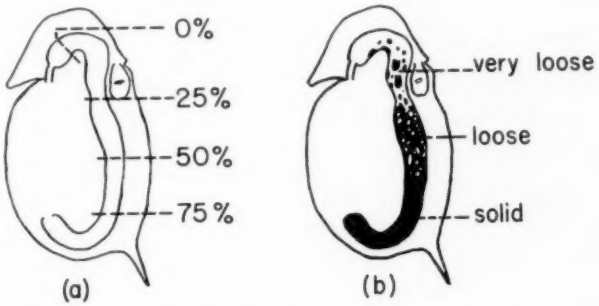

Fig. 3.—Diagram showing method of estimating (a) degree of evacuation of gut, and (b) consistency of contents.

about three minutes. With a piece of filter paper remove the solution in the flared portion of the observation tube, as the curvature of the glass in this portion makes observation difficult. The tubes can then be laid on the side without danger of loss of contents. Observations are best made with a capillary or dissecting microscope, using low enough power to provide a fairly large field as the active daphnia is then more easily followed.

The temperature should be constantly checked. If the room temperature is 25° C., no further provisions need be made. We have found that under lower temperature conditions the temperature can be easily regulated by placing the observation tubes in a small beaker, inserting a thermometer and controlling the temperature by moving the substage lamp nearer to or farther from the beaker. At temperatures higher than desired, the tubes may be placed on a folded piece of gauze which is in turn placed on a towel, and the whole saturated with cold water. By this means a temperature of 24.5° to 25.5° C. may be maintained.

Method of Observation.—Observations are made every ten minutes. For progressive evacuation studies the evacuation of the gut should be noted as 25 per cent, 50 per cent, 75 per cent, and 100 per cent. The estimation of the degree of evacuation is made according to the attached sheet (Fig. 3).

For the subdivision of the gut the following landmarks are employed: 0 per cent at the anterior portion of the tract, 25 per cent at the posterior end of the cardiac flexure, 50 per cent at the midpoint of the tract, and 75 per cent at the beginning of the curve of the postabdomen (Fig. 3*a*). To facilitate the recording of the amount of material in the gut, the condition of the tract may be noted as "very loose," "loose," and "solid" (Fig. 3*b*). When the contents present an unbroken, opaque appearance, it is termed "solid." When the tract becomes lighter in color and broken spaces begin to occur, it is called "loose." The appearance of small clumps of material separated by larger spaces indicates a "very loose" condition. When the contents no longer appear in the extreme posterior of the tract, it may be termed empty, even though small particles of food just ingested appear in the extreme anterior end. Recording the consistency of the contents in this manner enables the worker to obtain a fair estimate of the condition of peristaltic activity. An emptying of the tract which presents a solid mass of material moving through the intestine, indicates an increased rate in stool passage. If the solid material begins to loosen at the anterior end and show a tendency to continue loosening, it is almost certain that reverse peristalsis has set in. For quantitative comparison the time of 75 per cent evacuation is probably sufficient.

For work with the sparingly soluble bodies, such as phenolphthaleins, the saturated solution with excess has been used. Present indications are that in the case of more active agents, such as yellow phenolphthalein, the stimulation of peristalsis may actually be so strong as to inhibit evacuation. For this reason it may be necessary to work with dilutions and comparisons made of relative efficiency according to the dilution.

SUMMARY

A method of procedure for the use of *Daphnia magna* in the assay of catharties is herewith submitted. In order to secure comparable results it is important that a standard technique be established. We believe that the procedure described gives comparable results, although we are willing to admit the possibility and desirability of further improvement and refinement.

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DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

TUBERCULIN REACTION, Daily Variations in, Howe, J. S. Am. Rev. Tubere. 37: 264, 1938.

I. Range of Variation in Tuberculous and Control Subjects.

II. Relation to the Vascular Pressor Episode.

There is a definite correlation between the quantitative variations in the tuberculin reaction and the state of the peripheral vessels as measured by daily diastolic blood pressure levels. In this series of six subjects the correlation ranges from 77 per cent to 100 per cent.

Periods of peripheral vasoconstriction, characterized by increasing diastolic blood pressure, are accompanied by a decreased reaction to tuberculin, and in several instances by completely negative reactions to the smaller dose of tuberculin.

Periods of peripheral vasodilation, characterized by a falling diastolic blood pressure following a definite pressor peak, are accompanied by increased reaction to tuberculin. This exaggeration of the tuberculin reaction is most marked immediately following a marked pressor episode, and may subside with complete vasodilation.

In all subjects studied over the same period, the trend of the tuberculin reaction at the same time is roughly parallel; that is, the "peaks and troughs" of the curves for the different subjects coincide more or less closely. This is in accord with the work of Petersen who has shown that vasoconstriction and vasodilation, reflected in the blood pressure readings, are largely influenced by the meteorologic environment, particularly by the infall of polar air, which causes peripheral vasoconstriction, and is reflected as the "pressor episode."

The mechanism of these variations in the tuberculin reaction apepars to be one of periodic changes in vascular and tissue permeability, affecting the extent of local exudation in response to a definite dose of tuberculin. The increased permeability may also result in an actually increased sensitivity of the cells to tuberculin.

The vascular pressor episode may be reflected not alone in variations in the tuberculin reaction but also in clinical symptoms and chemical and serologic changes.

PHENOLPHTHALEIN STUDIES—Elimination of Phenolphthalein, Fantus, B., and Dyniewicz, J. M. J. A. M. A. **110**: 795, 1938.

Method of Determining Conjugated Phenolphthalein in Urine.—Heat 10 c.c. of urine and 10 c.c. of 10 per cent hydrochloric acid on a water-bath for from three to four hours to break up the conjugation. Allow the solution to cool. Extract with ether in from 15 to 20 c.c. portions until a small volume of the last washing shows no trace of phenolphthalein when tested with sodium hydroxide volumetric solution.

The combined, filtered ether portions containing the freed phenolphthalein are shaken with a small portion of tenth normal sodium hydroxide volumetric solution and allowed to stand until the mixture has completely separated. The alkaline solution is then drawn off into a second separator, and the process repeated until all the phenolphthalein is extracted. The combined alkaline solutions are than made acid with the smallest amount of hydrochloric acid and again extracted with ether.

The ether portions are made alkaline with tenth normal sodium hydroxide volumetric solution (the volume used depending on the amount of phenolphthalein present) and compared with the same volume of tenth normal sodium hydroxide volumetric solution, to which is added a known standard phenolphthalein solution to color equality, and the amount of phenolphthalein present is calculated.

Method of Determining Free Phenolphthalein in Urine.—Extract 10 c.c. of urine with ether in from 15 to 20 c.c. portions until a small volume of the last washing shows no trace of phenolphthalein when tested with tenth normal sodium hydroxide volumetric solution. The combined, filtered ether portions containing the phenolphthalein are shaken with tenth normal sodium hydroxide volumetric solution, the volume used depending on the amount of phenolphthalein present. It is advisable to make the extraction with as few separations as possible and to make the reading quickly because phenolphthalein in alkaline solution tends to decolorize. This solution is compared with the same volume of tenth normal sodium hydroxide volumetric solution, to which is added a known standard phenolphthalein solution to color equality, and the amount calculated.

INTOXICATION, ALCOHOLIC, Diagnosis and Medicolegal Implications, Selesnick, S. J. A. M. A. **110**: 775, 1938.

It is important to have definite criteria as a basis for the diagnosis of alcoholic intoxication in accidents involving individuals who have imbibed alcoholic beverages. The chemical determination of body fluid alcohol offers a scientific means of establishing whether or not an individual has imbibed alcohol and of estimating the degree of alcoholic intoxication. Blood as a medium for analysis is preferable to spinal fluid, urine, saliva, and expired air, because it contains a negligibly small amount of nonalcoholic oxidizable material, its alcoholic content represents the degree of alcohol saturation at the moment the blood sample is obtained, it is always available, and its extraction does not necessitate the active participation of the subject. There are sufficient scientific data to prove that subclinical intoxication —or alcoholic intoxication in the biologic case without any gross manifestations of drunkenness—can produce sufficient interference with psychomotor activity and neuromuscular coordination to render such an affected individual a potential public menace. Blood alcohol determinations can detect these degrees of alcoholic intoxication which ordinarily escape the detection of competent physicians. Criteria, therefore, must be established which include body fluid alcohol determinations as part of the diagnostic armamentarium.

SULFANILAMIDE: Para-aminobenzenesulfonamide, Absorption and Excretion, Method of Determination in Urine and Blood, Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C.: J. A. M. A. **108**: 953, 1937.

The authors present the following methods for the determination of sulfanilamide in the urine and blood. The method depends upon the presence of an amino group substituted in the benzol ring. Diazotization with nitrous acid in an acid solution produces a diazo compound with dimethyl-alpha-naphthylamine, a purplish-red azo dye estimated by colorimetry. The method has a sensitivity of 1 part to 20 million of water.

Reagents:

1. 0.1 per cent *freshly prepared* solution of sodium nitrite.
2. N/10 hydrochloric acid.
3. 95 per cent ethyl alcohol.
4. Dimethyl-alpha-naphthylamine 1 c.c. to 100 c.c. of alcohol.
5. Standard solution: 200 mg. of sulfanilamide per liter. From this stock solution standards are prepared containing 1.0, 0.5, and 0.2 mg. per cent. The standards are stable for several months if kept in the icebox.

Method for Urine:

1. Dilute the urine to contain 0.5 to 1.5 mg. per cent of sulfanilamide (with the amount ordinarily administered dilutions of 1:50, 1:100, or 1:200 suffice).
2. To 10 c.c. of diluted urine in a small flask, add 2 c.c. of HCl, 1 c.c. of sodium nitrite, 5 c.c. of alcohol, and 1 c.c. of naphthylamine. *Shake the flask after the addition of each reagent.*
3. Treat similarly 10 c.c. of the standard solution in a similar flask.
4. Allow both flasks to stand ten to fifteen minutes and match the solutions in a colorimeter.

Method for Blood:

1. While shaking, add 1 volume of blood to 9 volumes of alcohol, stopper the flask, allow to stand ten to fifteen minutes (or longer) and filter.

2. Transfer 10 c.c. of filtrate to a small flask and add 5 c.c. of water, 2 c.c. of HCl, and 1 c.c. of sodium nitrite.

3. Allow to stand three minutes and add 1 c.c. of naphthylamine.

4. In a similar flask add 1 c.c. of standard to 9 c.c. of alcohol and treat similarly.

Allow to stand ten to fifteen minutes, filter, and compare colorimetrically.

These determinations give the concentration of free sulfanilamide—not the conjugated form which, because of blocking of the amino group, does not give the color reaction until hydrolyzed by hydrochloric acid and heat.

SULFANILAMIDE, Estimation in Blood and Other Body Fluids, Proom, H. Lancet 234: 260, 1938.

Reagents:

1. 20 per cent aqueous trichloracetic acid.
2. 0.5 per cent sodium nitrite solution.
3. 1 per cent alcoholic solution of dimethyl-alpha-naphthylamine.

Method: (Applicable to blood, urine, milk, and spinal fluid.)

1. Dilute 1 c.c. of blood (or body fluid) with 2 c.c. of distilled water.
2. Add 1 c.c. of trichloracetic acid solution, mix, and filter through a small paper.
3. To 1 c.c. of filtrate, add 0.05 c.c. (1 drop) of sodium nitrite solution.
4. Allow to stand three minutes and add 0.5 c.c. of naphthylamine solution and mix by gentle shaking.
5. Allow to stand fifteen minutes and compare colorimetrically with standard solutions similarly treated.

Standards:

Prepared either from solutions of phenol red in phosphate buffer, or, for concentrations less than 0.01 mg. per c.c., from solutions of cobalt sulfate in dilute sulfuric acid. Standards are sealed in ½ by 4 inch test tubes of nonalkaline glass. Suitable ranges: 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025, and 0.001 mg. per c.c.

Method accurate to within 10 per cent.

TISSUE: Gum Dammar for Mounting Sections, Evans, N. Arch. Path. 25: 83, 1938.

Dissolve the resin in the "neutral histological (practical) xylene" specially prepared by the Eastman Kodak Company. This is done by placing the ingredients together in a large stoppered bottle in an incubator and stirring with a glass rod several times daily until the mixture is of a heavy syrupy consistency. This is usually accomplished in about a week's time. Then strain through four layers of clean gauze and store in a dark bottle. If the resultant solution is found to be too thin for mounting purposes, a thicker consistency can be secured by placing the unstoppered bottle in an incubator for evaporation until it is of the right consistency. A satisfactory solution is considerably thinner and much less sticky than the preparations of Canada balsam generally used, and can be applied to the slide quickly.

The following advantages are noted for this method:

Slides mounted with gum dammar are much cleaner, dry quicker, will not stick together if allowed to dry for a few months before storing in contact with other slides, do not discolor, and do not fade.

An important advantage is the possibility of storing slides in compact arrangements, since they have no tendency to stick to one another, thus saving much space and expense in providing cabinets for the permanent filing of the slides.

Prepared solutions of gum dammar ready for use can be purchased from laboratory supply houses.

TUBERCULOSIS: The Weltmann Serocoagulation Reaction, Levinson, S. A., and Klein, R. I. Am. Rev. Tubere. 37: 200, 1938.

The Weltmann serum coagulation test is of importance in distinguishing between exudative and productive changes. The technique is simple, and the method is described. In general, acute inflammatory and exudative conditions give a shortened coagulation band or a shift to the left. In chronic diseases, characterized by fibrotic changes, in the healing stage of acute infection, and in parenchymal liver damage, the coagulation band tends to be lengthened, or results in a shift to the right. The coagulation band does not always parallel the sedimentation rate. In tuberculosis the Weltmann coagulation test is of importance because it can be used as a guide in the course of and in the prognosis of the disease. It reflects tissue changes that occur in the body. Exudative changes in tuberculosis are manifested by a shortening of the coagulation band, while fibrotic changes are accompanied by a prolonged coagulation band. When exudative and fibrotic changes occur in the lung at the same time, as happens frequently in tuberculosis, the Weltmann reaction may not be of great assistance. The two divergent processes occurring at the same time result in the coagulation band striking the normal zone. In miliary tuberculosis, peritoneal tuberculosis, and in meningeal tuberculosis, the Weltmann reaction gives a shortening of the coagulation band in the exudative zone. Isolated bone tuberculosis is accompanied by a normal or prolonged coagulation band and is reflected in the fibrotic zone. There is a correlation between the blood pH and the Weltmann reaction, but the authors believe that both of these changes may be directly related to the underlying process. They have applied the Weltmann test not only to tuberculosis in the minimal, moderately advanced, and far advanced stages, but also to the complications that occur in pulmonary disease. They have also employed the Weltmann reaction and compared it with the sedimentation rate in several examples of various types of tuberculosis over a prolonged period of time. The results are charted in the text. Although unable at the present to explain the mechanism of the Weltmann serum coagulation reaction, they believe that this test is of importance in the diagnosis and prognosis of disease, particularly in tuberculosis.

The technique follows:

From a stock solution of 10 per cent calcium chloride ($CaCl_2.6H_2O$), ten dilutions consisting of 0.1 per cent, 0.009 per cent, 0.08 per cent, 0.07 per cent, 0.06 per cent, 0.05 per cent, 0.04 per cent, 0.03 per cent, 0.02 per cent, and 0.01 per cent are made up. These dilutions are numbered from one to ten, beginning with the strongest concentration. Ten small test tubes, such as are used in the Wassermann reaction, are placed in a metal rack and numbered in order from 1 to 10. Into each tube is pipetted 5 c.c. of the similarly numbered calcium chloride solution and 0.1 c.c. of unhemolyzed serum. The tubes are then shaken so that the contents are well mixed and placed in a boiling water bath for fifteen minutes. They are then removed and the reaction can be immediately determined. The contents of the tubes may be clear, faintly opalescent, turbid, or there may be a flocculation. There is usually a sharp and easily noted difference between flocculation and turbidity.

The number of tubes in which coagulation occurs is designated by Weltmann as the coagulation band (C.B. of that particular serum). If there is a very slight or doubtful flocculation in one tube the reaction is interpreted as being intermediate between that tube and the one before it. In normal serum the first 6 tubes usually show flocculation. Sometimes there is a slight or doubtful flocculation in the seventh tube. The normal coagulation band is, therefore, 6 to 6½, and is remarkably constant. This means that normal human serum diluted 50 times with calcium chloride solution and heated in a boiling water-bath for fifteen minutes will not coagulate or flocculate if the concentration of the calcium chloride is less than 0.04 per cent. If the coagulation band is less than 6, Weltmann speaks of the reaction as showing a shift to the left, whereas, if the coagulation band is 7 or greater, the reaction indicates a shift to the right.

NEPHROSIS: Metabolic Study of Five Children With the Nephrotic Syndrome, Mitchell, A. G., Rittershofer, C. R., Wang, C. C., Kaucher, M., Wing, M., and Hogden, C. Am. J. Dis. Child. 55: 27, 1938.

Metabolic studies on five nephrotic children, two boys and three girls, between the ages of 3 and 16 years, were made during periods lasting from forty-two to two hundred and fifty-five days. In this report the clinical data, the effect of various treatments, and the records of excretion of urinary protein, studies of the blood, basal metabolism, and the energy exchange are presented.

The effects of diuretics and dietary treatment varied greatly in producing temporary changes. However, no prolonged general improvement, including disappearance of edema, was accomplished with any procedure.

The protein content (albumin and globulin) of the serum, its nonprotein nitrogen content, and the cholesterol content of the blood were determined from time to time for the five patients. The values were those characteristic for the nephrotic syndrome; i.e., there were a low total protein content, a reversal of the ratio of albumin to globulin, a normal value for serum nonprotein nitrogen, except in two patients in whom the nephrotic syndrome was complicated, and a high content of blood cholesterol. The concentration of hemoglobin and the cell counts showed no definite changes.

The basal metabolism and the energy exchange are discussed in relation to age, height, and weight. The values are compared with data reported for normal children. The values for basal metabolism of all patients were within normal limits according to the standard of Benedict and Talbot and, with one exception, to the standard of Harris and Benedict. According to Dreyer's standard the heat production of all patients except one was low. From the determinations of caloric intake and caloric loss, calculations were made for the percentage absorption of energy and the expenditure of energy for growth, digestion, and activity. These values agreed with data obtained for normal children. This indicates that the energy exchange of a nephrotic patient is normal.

SULFANILAMIDE

Mall, J. W., and Smith, J. T. (J. A. M. A. **110**: 439, 1938) report an actual lowering of the capacity of the blood for oxygen following the use of this drug.

Swift, H. F., Moen, J. K., and Hirst, G. K. (J. A. M. A. **110**: 426, 1938) conclude that the toxic action of sulfanilamide in active rheumatic fever so far outweighs the beneficial therapeutic effect that its use in this disease does not seem justified.

Bilger, J. A., Clifton, W. M., and Werner, M. (J. A. M. A. **110**: 343, 1938) report that sulfanilamide seemed to cause a depression of the white blood cells even to a point at which a leucopenia developed.

Agranulocytosis or granulopenia did not occur with this depression or leucopenia.

The action of sulfanilamide seems to be independent of the leucocytes in that it does not produce an increase in the total leucocytes or in the proportion of the polymorphonuclear cells.

Cyanosis occurred in seven cases but spectroscopic examination of the blood specimens in these cases did not reveal bands of sulfhemoglobin or methemoglobin.

Sulfanilamide is a very effective drug in beta hemolytic streptococcus infections.

Frequent blood cell determinations should accompany sulfanilamide therapy.

Osgood (J. A. M. A. **110**: 349, 1938) discusses the mode of action and concludes that the major action of sulfanilamide on the beta hemolytic streptococcus seems to be neutralization of the toxins. Either because of this action or incidentally it also decreases the rate of cell division of this organism. It appears not to kill these organisms directly, al-

though it does permit the bactericidal properties of human serum and to some extent phagocytosis by leucocytes to kill organisms which they otherwise would be unable to kill. It has no direct effect on phagocytosis.

The effective concentration of sulfanilamide would appear to be about 1:100,000 or only one tenth of that now ordinarily maintained in the blood stream, but this experimental observation requires confirmation by carefully controlled experiments on large numbers of human infections before it is justifiable to employ smaller dosages in dangerously ill patients. Sulfanilamide in concentrations even greater than those generally employed clinically does not appear to have direct toxic action on the nucleated cells of the majority of bloods or marrows. This does not exclude the occurrence of an occasional idiosyncrasy in the reactions of these cells such as is known to occur for other benzene ring drugs.

The possible effectiveness of and the mode of action of sulfanilamide on all other organisms known to produce human disease should be determined by the methods here described as soon as possible. Cultures of human marrow should aid materially in the study of the mode of action of both noxious and therapeutic agents.

Another case of fatal granulocytopenia is reported by **Schwartz, W. F., Garvin, C. F., and Kaletsky, S.** (J. A. M. A. **100**: 368, 1938) and still another is recorded by **Berg, S., and Holtzman, M.** (J. A. M. A. **110**: 370, 1938).

REVIEWS

Books and Monographs for Review should be sent direct to the Editor, Dr. Warren T. Vaughan, Professional Building, Richmond, Va.

The Control of Syphilis and Other Infectious Diseases*

THIS volume presents the fifth series of Abraham Flexner lectures, presented in the School of Medicine of Vanderbilt University.

In view of the widespread publicity given to the campaign against syphilis and the frequent reference and the similar campaign conducted in Denmark, the first lecture "Control of Venereal Disease in Denmark With Special Reference to Syphilis," which, incidentally, was under Dr. Madsen's direction, will be read with great interest.

It is of particular interest to note in the account given in this lecture, that the attempt at control of syphilis through state agencies in Denmark began as long ago as 1774 and that the results widely quoted had their inception in a law promulgated in 1906.

The second lecture is concerned with the mechanism of bacterial infection; the third discusses tuberculosis in Denmark; the fourth, the influences of seasons on infection; and the final lecture is devoted to whooping cough.

All these lectures are of scholarly quality and constitute real and valuable contributions to the subjects in question. This book may well be added to the physician's library.

The Diary of a Surgeon in the Years 1751-1752†

THIS is not a book to be started if there is an important appointment in the office, for once begun this book can only be laid down with reluctance.

There is some justification for sprinkling the proverbial grain of salt on publishers' announcements but that on the jacket of this book is not only true to the letter but, if anything, a little conservative.

It says, "This is one of those intriguing books which defies classification. It can be enjoyed as a vivid reconstruction of old medical practice, it can be read as a tale of high adventure on the sea, or it can be savored as a delightful piece of writing with a distinctive Pepysian flavor. Whatever, it is, it makes thrilling reading—an unusual treasure of a book which does more to throw light on past days than reams of histories."

This is exactly that sort of a book. If you own it you won't want to lend it because, if you had borrowed it you would have to return it. If you do not read it, you will miss a book undoubtedly destined to become a classic. If, for any reason, you have decided not to buy any more books for a while, do not open this one. You will not be able to resist it if you do.

For the benefit of the general reader a glossary of scientific names and a glossary of terms is included—wisely, for these rollicking pages will have a general and widespread appeal.

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*Lectures on the Epidemiology and Control of Syphilis, Tuberculosis, and Whooping Cough, and Other Aspects of Infectious Disease. By Thorvald Madsen, M.D., Director of the State Serum Institute of Denmark, Copenhagen; Chairman of the Health Committee of the League of Nations. Cloth, 216 pages, 21 figures, $3.00. Williams & Wilkins Co., Baltimore, Md.

†The Diary of a Surgeon in the Year 1751-1752. By John Knyveton, Licentiate of the Society of Apothecaries; Doctor of Medicine of the University of Aberdeen; Teacher of Midwifery and Man-Midwife in Infirmary Hall; Surgeon's Mate H.M.S. Lancaster. Edited and Transcribed by Ernest Gray. Cloth, 332 pages, 7 illustrations, $2.50. D. Appleton-Century Co., New York, N. Y.

Handbook of Medicine*

ANY book reaching a tenth edition must have proved itself useful and of value to those to whom it is addressed. This volume proves the rule. The present edition has been extensively revised and much new matter added throughout.

It may be predicted with confidence that this edition will meet the same cordial reception awarded to its predecessors.

The Hair and Scalp†

IN A REVIEW of the first edition of this book it was predicted that it would receive a cordial reception. The necessity for a second edition within two years is, hence, no surprise. This little volume will be found eminently practical, and a most useful addition to the practitioner's bookshelf. It can be recommended as an important contribution to a neglected subject.

The Medicolegal Aspects of the Ruxton Case‡

THIS is a volume of extraordinary interest. While Professor Glaister has achieved an international reputation as a medicolegal expert, it is doubtful whether his previous experience included a case with so many bizarre aspects, or presented so many difficulties as the case the story of which is here related.

The problem presented to the experts for the crown in the Ruxton case was the reconstruction and identification of two bodies, represented by over seventy gruesome fragments.

The bodies, those of Mrs. Ruxton and her maid-servant, had not only been dismembered but subjected to partial dissection and extraordinary mutilation in an effort to render identification impossible. To this task new methods were brought, in addition to those familiar to legal medicine, as a result of which identification was indubitably established, and the murderer, Dr. Ruxton, duly convicted and executed.

The book details through text and numerous illustrations the methods used. To the pathologist, the police surgeon, the lawyer, and all concerned with medicolegal problems this volume will prove a classic of its kind.

Vade Mecum of Medical Treatment§

THIS is a handbook the aim of which is to provide an account of the treatment of those conditions most commonly seen in general practice. To facilitate ready reference the diseases noted are arranged in alphabetical order. An index makes the contents of the book readily accessible.

The methods advocated throughout the book are either those with which the author has had personal experience, or those recommended by standard authorities.

As a ready reference, this book should prove useful to the practitioner at large.

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*Wheeler and Jack's Handbook of Medicine. Revised by John Henderson, M.D., M.R.C.P., Physician, Glasgow Infirmary; Professor of Medicine, St. Mungo's College, Glasgow; Hon. Lecturer in Clinical Medicine, University of Glasgow; Examiner in Medicine for Fellowship of R.F.P.S. (Glasgow); Lecturer in Medicine and Physician to Nurses, Glasgow Royal Infirmary. Leatherette, ed. 10, 703 pages, 27 figures, $4.00. William Wood & Co., Baltimore, Md.

†Hair and Scalp. By Agnes Savill, M.A., M.D., M.R.C.P.I., Consulting Physician to Fitzroy Square Skin Hospital; Formerly Physician to St. John's Hospital for Skin Diseases, Leicester Square, etc. Cloth, ed. 2, 309 pages, 54 illustrations, $4.75. William Wood & Co., Baltimore, Md.

‡The Medicolegal Aspects of the Ruxton Case. By John Glaister, M.D., D.Sc., Barrister-at-Law, Regius Professor of Forensic Medicine, University of Glasgow, and James Couper Brash, M.A., M.D., F.R.C.S., Ed., Professor of Anatomy, University of Edinburgh. Cloth, 284 pages, 174 illustrations, $6.00. William Wood & Co., Baltimore, Md.

§Vade Mecum of Medical Treatment. By W. Gordon Sears, M.D., M.R.C.P., Deputy Medical Superintendent, St. Charles Hospital, London. Cloth, 368 pages, $4.00. William Wood & Co., Baltimore, Md.